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REMARKS

Amendments

Claims 1-35 have been canceled and claims 36, 42, 44 and 45 have been amended. Upon entry of the amendment, claims 36-48 will be pending. Support for the added claims can be found in the specification, for example, page 7, lines 25-28; page 19, line 8 through page 2, line 22; page 52, lines 18-29; page 53, line 1 through page 54, line 5; the Figures; and in the claims as originally filed.

The foregoing amendments are made solely to expedite prosecution of the application and are not intended to limit the scope of the invention. Further, the amendments to the claims are made without prejudice to the pending or now canceled claims or to any subject matter pursued in a related application. The Applicant reserves the right to prosecute any canceled subject matter at a later time or in a later filed divisional, continuation, or continuation-in-part application.

Specification

The Examiner has objected to the specification on the basis that the disclosure contains improper incorporation of subject matter by reference to U.S non-provisional and provisional applications.

The specification has been amended to update the status of the originally cited application references. No new matter has been introduced.

Rejections

Rejections under 35 U.S.C. § 101

The Examiner has rejected claims 30-35 because the claimed invention is allegedly not supported by either a specific or substantial asserted utility or a well-established utility.

Applicant respectfully traverses the rejection. Amended claim 36 is drawn to a transgenic mouse whose genome comprises a null limulus clotting factor protease-like allele.

1. Utility Guidelines

According to 35 U.S.C. § 101, "[w]hoever invents . . . any new and useful . . . composition of matter may obtain a patent therefore. . . . "

Under the Patent Office's Utility Requirement Guidelines:

If at any time during the examination, it becomes readily apparent that the claimed invention has a well-established utility, do not impose a rejection based on lack of utility. An invention has a well-established utility if (i) a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention (e.g., properties or applications of a product or process), and (ii) the utility is specific, substantial, and credible.

. . .

If the applicant has asserted that the claimed invention is useful for any particular practical purpose (i.e., it has a "specific and substantial utility") and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility.

(emphasis added)(MPEP § 2107, II (A)(3); II (B)(1)).

The standard for "credible" is defined as:

. . . whether the assertion of utility is believable to a person of ordinary skill in the art based on the totality of evidence and reasoning provided. An assertion is credible unless (A) the logic underlying the assertion is seriously flawed, or (B) the facts upon which the assertion is based are inconsistent with the logic underlying the assertion.

(MPEP 2107.02, III(B)(emphasis added).

According to the Patent Office's own guidance to Examiners:

Langer and subsequent cases direct the Office to presume that a statement of utility made by an applicant is true. [citations omitted] . . . Clearly, Office personnel should not begin an evaluation of utility by assuming that an asserted utility is likely to be false.

Compliance with 35 U.S.C. 101 is a question of fact [citations omitted]. Thus, to overcome the presumption of truth that an assertion of utility by the applicant enjoys, Office personnel must establish that it is more likely than not that one of ordinary skill in the art would doubt (i.e., "question") the truth of the statement of utility. . . . To do this, Office personnel must provide evidence sufficient to show that the statement of asserted utility would be considered "false" by a person of ordinary skill in the art.

(MPEP 2107.02, III(A)(emphasis added).

Rejections under 35 U.S.C. 101 have been rarely sustained by federal courts. Generally speaking, in these rare cases, the 35 U.S.C. 101 rejection was sustained either because the applicant failed to disclose any utility for the invention or asserted a utility that could only be true if it violated a scientific principle, such as the second law of thermodynamics, or a law of nature, or was wholly inconsistent with contemporary knowledge in the art. *In re Gazave*, 379 F.2d 973, 978, 154 USPQ 92, 96 (CCPA 1967). Special care therefore should be taken when assessing the credibility of an asserted

therapeutic utility for a claimed invention. In such cases, a previous lack of success in treating a disease or condition, of the absence of a proven animal model for testing the effectiveness of drugs for treating a disorder in humans, should not, standing alone, serve as a basis for challenging the asserted utility under 35 U.S.C. 101.

(MPEP 2107.02, III(B)(emphasis in original and added). The Guidelines additionally provide that:

> There is no predetermined amount or character of evidence that must be provided by an applicant to support an asserted utility, therapeutic or otherwise. Rather, the character and amount of evidence needed to support an asserted utility will vary depending on what is claimed (citations omitted), and whether the asserted utility appears to contravene established scientific principles and beliefs. (citations omitted). Furthermore, the applicant does not have to provide evidence sufficient to establish that an asserted utility is true "beyond a reasonable doubt." (citations omitted). Nor must an applicant provide evidence such that it establishes an asserted utility as a matter of statistical certainty. Nelson v. Bowler, 626 F.2d 853, 856-57, 206 USPQ 881, 883-84 (CCPA 1980)(reversing the Board and rejecting Bowler's arguments that the evidence of utility was statistically insignificant. The court pointed out that a rigorous correlation is not necessary when the test is reasonably predictive of the response).

(MPEP 2107.02, VII)(emphasis added).

Thus, according to Patent Office guidelines, a rejection for lack of utility may not be imposed where an invention has a well-established utility or is useful for any particular practical purpose. An assertion of utility is presumed to be true. The burden is on the Examiner to show that one of ordinary skill would find the asserted utility to be false. The present invention satisfies either standard.

2. Well-Established Utility

According to 35 U.S.C. § 101, "[w]hoever invents . . . any new and useful . . . composition of matter may obtain a patent therefore. . . . "

Under the Patent Office's Utility Requirement Guidelines:

> If at any time during the examination, it becomes readily apparent that the claimed invention has a well-established utility, do not impose a rejection based on lack of utility. An invention has a well-established utility if (i) a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention (e.g., properties or applications of a product or process), and (ii) the utility is specific, substantial, and credible.

Applicant submits that it cannot be reasonably argued, in light of arguments of record, that a person of ordinary skill in the art would not immediately appreciate why the invention is useful. Examiner notes that "it was scientifically well-known to knock out a gene to determine its function or what will happen when the gene is not expressed" (page 8). The Examiner further notes that "it is clear from all of the art provided by Applicants that knockout mice are used to elucidate gene function" (page 9). Thus, it is not debated that a person of ordinary skill in the art would immediately appreciate why the invention is useful: for determining gene function.

3. Specific

The Examiner states that the asserted use is not specific (page 12).

According to the MPEP, "specific utility" means "specific" to the subject matter claimed as compared to a "general utility" that would be applicable to the broad class of the invention (MPEP 2107.01). Use of the protease-like -/- mouse to study the function of the protease-like gene and the association of the protease-like gene with, for example, pain and seizures, is specific to this mouse. Even if there were many other genes associated with these phenotypes, only a protease-like knockout mouse (as opposed to all other knockout mice) would be used to study the specific role of this gene in pain and seizures. The Examiner is respectfully requested to explain (1) how the asserted utility of determining the function of the protease-like gene would be applicable to all other knockout mice; and (2) how the asserted use of studying the association of the protease-like with pain and seizures would be applicable to all other knockout mice.

In addition, the mice within the scope of claim 42 contain a *lacZ* gene. The Examiner acknowledges that this "is a widely used technique to generate mouse knockouts by inserting a visible reporter into an endogenous gene" (page 12). However, the Examiner argues that the asserted use is not specific and is a general utility that applies to any knockout mouse.

Applicant strongly disagrees. As noted by the Examiner, and as is well understood in the art, the *lacZ* gene is inserted into the endogenous gene. In this case, the *lacZ* gene was inserted into the locus of the protease-like gene. Expression is driven by the endogenous promoter. Expression of the *lacZ* gene indicates where the protease-like gene is expressed. This use is specific for this mouse – knockout mice in general cannot be used for this purpose. The

Examiner is requested to explain how all other knockout mice would be used to study expression of the lumulus clotting factor protease-like gene.

4. Substantial

According to the MPEP, under the section entitled "Substantial Utility":

> A "substantial utility" defines a "real world" use. Utilities that require or constitute carrying out further research to identify or reasonably confirm a "real world" context of use are not substantial utilities. . . . the following are examples of situations that require or constitute carrying out further research to identify or reasonably confirm a "real world" context of use and, therefore, do not define "substantial utilities":
>
> (A) Basic research such as studying the properties of the claimed product itself or the mechanisms in which the material is involved;
>
> Office personnel must be careful not to interpret the phrase "immediate benefit to the public" or similar formulations in other cases to mean that products or services based on the claimed invention must be "currently available" to the public in order to satisfy the utility requirement. See, e.g., Brenner v. Manson, 383 U.S. 519, 534-35, 148 USPQ 689, 695 (1966). Rather, **any reasonable use** that an applicant has identified for the invention that can be viewed as providing a public benefit **should be accepted as sufficient,** at least with regard **to defining a "substantial" utility.**
>
> (MPEP § 2107.01 I)(emphasis added).

The MPEP additionally provides:

> Some confusion can result when one attempts to label certain types of inventions as not being capable of having a specific and substantial utility based on the setting in which the invention is to be used. One example is inventions to be used in a research or laboratory setting. Many research tools such as gas chromatographs, screening assays, and nucleotide sequencing techniques have a clear, specific and unquestionable utility (e.g., the are useful in analyzing compounds). An assessment that focuses on whether an invention is useful only in a research setting thus does not address whether the invention is in fact "useful" in a patent sense. Instead, Office personnel must distinguish between inventions that have a specifically identified substantial utility and inventions whose asserted utility requires further research to identify or reasonably confirm. Labels such as "research tool," "intermediate" or "for research purposes" are not helpful in determining if an applicant has identified a specific and substantial utility for the invention.

(MPEP 2107.01, I)

A use is not substantial where further research is required to identify any use. This is not the case in the present application. Knockout mice have a well-known use in the study of gene function. In the present case, the instant invention does not require further research to establish a utility. Applicant has determined that the protease-like gene is associated with, for example, pain and seizures. No further research is required to establish any use. The invention has a "real world use" – as demonstrated by the delivery of the claimed invention to at least one large pharmaceutical company. Whether additional research is required to identify therapeutic agents targeting the protease-like gene or to further characterize the function of the protease-like gene is irrelevant to whether the claimed invention has satisfied the utility requirement.

The Examiner has not responded to Applicant's previous argument that a commercial sale represents a substantial, real-world use. The Examiner has also not challenged the commercial sale itself. Although evidence was not required by the Examiner, in support of such commercial use, attached hereto is a Declaration from Robert Driscoll stating that knockout mice obtained from Deltagen are used for determining gene function and for drug discovery purposes – both uses which are clearly stated throughout the specification.

The Examiner asserts the claimed mice are not useful as research tools because using a product for further research is not a "substantial utility."

Applicant does not agree. First, it is wholly untrue that further research is required in order to confirm the utility of the claimed mouse in determining the function of protease-like . The value of knockout mice in determining gene function is well established and accepted in the art. This is demonstrated by the references cited previously. The Examiner has failed to provide sufficient factual support for the position that it is more likely than not that a person of skill in the art would doubt that Applicant's asserted utility is specific and substantial, which is the standard for establishing a *prima facie* case. See MPEP § 2107.02, IV.

Second, Applicant is claiming a transgenic mouse, and not the protease-like or nucleic acid sequence. The Examiner must differentiate between the utility of the transgenic mouse and the utility of the target gene. "The claimed invention is the focus of the assessment of whether an applicant has satisfied the utility requirement." (MPEP 2107.02, I) That the claimed transgenic mouse can be used in a research setting to further characterize the protease-like gene

does not mean that the mouse lacks patentable utility. Further characterization (involving "basic research") of the mouse itself is not necessary in order to confirm its utility in studying the function of the protease-like gene.

The Examiner notes that "it is clear from all of the art provided by Applicants that knockout mice are used to elucidate gene function, which is not considered a substantial utility" (page 9).

According to the MPEP:

> **any reasonable use** that an applicant has identified for the invention that can be viewed as providing a public benefit **should be accepted as sufficient,** at least with regard to defining a "substantial" utility.

Certainly providing an *in vivo* model for studying the function of the limulus clotting factor protease-like gene is a reasonable use.

In addition, the MPEP specifically cautions Examiners not to get confused by labeling inventions as research tools:

> Office personnel must distinguish between inventions that have a specifically identified substantial utility and inventions whose asserted utility requires further research to identify or reasonably confirm. Labels such as "research tool," "intermediate" or "for research purposes" are not helpful in determining if an applicant has identified a specific and substantial utility for the invention.

The Examiner seeks to distinguish the claimed invention from a gas chromatograph by arguing that the "claimed invention is not a general tool for analyzing other samples and, at most, serves to study the function of a single gene" (page 8).

Applicant disagrees. One of the asserted uses of the claimed invention is determining the specificity of an agent by measuring a physiological response of the animal to the agent and comparing the physiological response of such animal to a control animal (see, e.g., claim 46). The Examiner is requested to explain why this is not a tool for analyzing other samples (e.g., an agonist or agent capable of up regulating the gene, etc).

Applicant submits that the Examiner has done what the MPEP specifically cautions against, by providing: "[a]n assessment that focuses on whether an invention is useful only in a research setting thus does not address whether the invention is in fact "useful" in a patent sense."

The Examiner argues that scientific "utility" is not the same as "patentable utility" or a "well-established" utility.

Applicant does not agree. According the Utility Guidelines,

> If the applicant has asserted that the claimed invention is useful for any particular practical purpose (i.e., it has a "specific and substantial utility") and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility.

As acknowledged by the Examiner, the use of knockout mice to study gene function is well-known – i.e., the mouse has scientific utility. If the asserted use is considered credible and accepted by the scientific community, how can such a use not be regarded as substantial? Applicant submits that if a claimed invention has scientific utility, it necessarily follows that the invention has patentable utility.

5. In re Brana

The Examiner also argues that the fact pattern in *Brana* does not apply to the fact pattern of the instant application because in *Brana* the specification did disclose a specific and substantial use for the claimed compound.

Applicant submits that the legal principles as well as the facts of *Brana* are applicable to the present case. In *Brana*, the Board held that the applicant's specification failed to disclose a specific disease against which the claimed compounds were useful. The Federal Circuit reversed and held that the mouse tumor model represented a specific disease against which the compounds were effective. In the present case, the Examiner has argued that Applicant failed to demonstrate a link between the protease-like gene and any of the recited phenotypes. It is Applicant's position that a mouse demonstrating, for example, increased sensitivity to pain and increased susceptibility to seizure, is sufficient to establish the animal's use as a model for pain and seizures. As in *Brana*, Confirmation of the phenotype in humans is unnecessary.

The Examiner argues that in *Brana*, the use of the compounds was "overlooked" by the PTO.

Applicant respectfully submits that the Examiner has misread the case, as the PTO did not overlook the asserted use:

> Applicants' specification, however, also states that the claimed compounds have "a better action and a better action spectrum as antitumor substances" than known

compounds, specifically those analyzed in Paull. As previously noted, see supra note 4, Paull grouped various benzo [de]isoquinoline-1,3-diones, which had previously been tested in vivo for antitumor activity against two lymphocytic leukemia tumor models (P388 and L1210), into various structural classifications and analyzed the test results of the groups (i.e. what percent of the compounds in the particular group showed success against the tumor models). Since one of the tested compounds, NSC 308847, was found to be highly effective against these two lymphocytic leukemia tumor models, 14 applicants' favorable comparison implicitly asserts that their claimed compounds are highly effective (i.e. useful) against lymphocytic leukemia. An alleged use against this particular type of cancer is much more specific than the vaguely intimated uses rejected by the courts in Kirk and Kawai. See, e.g., Cross v. Iizuka, 753 F.2d at 1048, 224 USPQ at 745 (finding the disclosed practical utility for the claimed compounds -- the inhibition of thromboxane synthetase in human or bovine platelet microsomes -- sufficiently specific to satisfy the threshold requirement in Kirk and Kawai.)

The Commissioner contends, however, that P388 and L1210 are not diseases since the only way an animal can get sick from P388 is by a direct injection of the cell line. The Commissioner therefore concludes that applicants' reference to Paull in their specification does not provide a specific disease against which the claimed compounds can be used. We disagree.

(*Brana* at 1440). Thus, contrary to the Examiner's characterization of *Brana*, the PTO was aware of the asserted use against the mouse tumor lines but did not find the use specific – as in the present case.

The court went on:

The ultimate issue is whether the Board correctly applied the Section 112 Para.1 enablement mandate and its implicit requirement of practical utility, or perhaps more accurately the underlying requirement of Section 101, to the facts of this case. As we have explained, the issue breaks down into two subsidiary issues: (1) whether a person of ordinary skill in the art would conclude that the applicants had sufficiently described particular diseases addressed by the invention, and (2) whether the Patent Act supports a requirement that makes human testing a prerequisite to patentability under the circumstances of this case.

The first subsidiary issue, whether the application adequately described particular diseases, calls for a judgment about what the various representations and discussions contained in the patent application's specification would say to a person of ordinary skill in the art. We have considered that question carefully, and, for the reasons we explained above in some detail, we conclude that the Board's judgment on this question was erroneous. Our conclusion rests on our understanding of what a person skilled in the art would gather from the various art cited, and from the statements in the application itself. We consider the Board's error to be sufficiently clear that it is reversible whether viewed as clear error or as resulting in an arbitrary and capricious decision.

> The second subsidiary issue, whether human testing is a prerequisite to patentability, is a pure question of law: what does the practical utility requirement mean in a case of this kind. Under either our traditional standard or under the APA standard no deference is owed the Agency on a question of law, and none was accorded.
>
> If the question concerning the standard of review, raised by the Commissioner, is to be addressed meaningfully, it must arise in a case in which the decision will turn on that question, and, recognizing this, the parties fully brief the issue. This is not that case. We conclude that it is not necessary to the disposition of this case to address the question raised by the Commissioner; accordingly, we decline the invitation to do so.

(*Brana* at 1443-44). The court's position is reflected in the MPEP: if an "assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility" (MPEP § 2107, II (A)(3); II (B)(1)). If it is well known to those skilled in the art that knockout mice are useful for studying gene function, then those skilled in the art would certainly regard such use as credible, specific and substantial. Nothing more is required to satisfy the statutory requirement. Applicant submits that, as in *Brana*, one skilled in the art would find the asserted use credible, substantial and specific.

The Examiner argues that the "mice exhibit a phenotype that fails to be correlated to the function of the limulus clotting factor C" and therefore the mice do not have "utility" (page 11).

Applicant requests the Examiner to provide the legal basis for the rejection. Is the Examiner's comment based on lack of credibility, specificity or substantiality? The phenotypes were observed by comparing the transgenic mice with wild-type control mice of identical background. As reported in the specification,

> Homozygous mice showed a statistically significant decrease in their response latency to the hot plate test, relative to wild-type animals. Specifically, when compared to age- and gender-matched wild-type control mice, homozygous mutant animals were significantly different from wild-types in their response to the hot plate test. The mutant animals showed a statistically significant decrease in the amount of time to lick their hindpaw when placed on the hot plate at 55°C, exhibiting an increased pain threshold.

(Example 1). Applicant reminds the Examiner that assertions made in a specification are presumed to be truthful.

6. Summary

In summary, Applicant submits that the claimed transgenic mouse, regardless of any disclosed phenotypes, has inherent and well-established utility in the study of the function of the

gene, and thus satisfies the utility requirement of section 101. Moreover, Applicant believes that the transgenic mice are useful for studying the function of the target protease gene with respect to the cited phenotypes as well as studying gene expression; and are therefore useful for a specific practical purpose that would be readily understood by and considered credible by one of ordinary skill in the art.

In light of the amendments and arguments set forth above, Applicant does not believe that the Examiner has properly established a *prima facie* showing that establishes that it is more likely than not that a person of ordinary skill in the art would not consider that any utility asserted by the Applicant would be specific and substantial. (*In re Brana*; MPEP § 2107).

Withdrawal of the rejections is respectfully requested.

Rejection under 35 U.S.C. § 112, first paragraph

The Examiner has rejected claims 36-48 under the first paragraph of 35 U.S.C. § 112 because one skilled in the art would allegedly not know how to use the claimed invention as a result of the alleged lack of either a specific or substantial asserted utility or a well-established utility for the reasons set forth in the utility rejection. Applicants respectfully traverse the rejection. For the reasons set forth above, it is Applicant's position that the claimed invention satisfies the utility requirement. Therefore, one skilled in the art would know how to use the invention.

In addition, the Examiner has rejected claims 36-48 for allegedly failing to comply with the enablement requirement.

The Examiner argues that the specification does not teach that the control with which the claimed mice were compared to were strain matched.

First, the specification and claims clearly recite the phenotypes were observed by comparing the claimed mice with wild-type control mice. It well known in the art to compare the transgenic mice with controls of the same background.

Second, attached hereto is a Declaration from John Burke, Attorney of Record, stating that the transgenic mice were in fact compared controls of identical background.

With regard to the added claims, the Examiner argues that specification does not teach how to make a null allele; that phenotype is unpredictable; and that gene disruptions "can" lead to hypomorphic and hypermorphic alleles.

First, the general rule is that disruption of the coding sequence by a positive selection marker, as taught in the specification, will result in a null allele, which by definition involves ablation of gene function (see, for example, Hasty et al., *Gene Targeting, Principles, and Practice in Mammalian Cells* in Gene Targeting: *A Practical Approach*, ed. Joyner, Oxford University Press 2000, p. 5)(copy attached). As shown in Figure 2B, the targeting vector was introduced by homologous recombination into the protease-like locus. As shown, the targeting vector contains exogenous DNA comprising *lacZ* and *Neo*^{r} genes. Homologous recombination was confirmed using both 3' and 5' PCR and southern blot analysis. According to Example 1: "[t]he targeting construct was introduced into ES cells derived from 129/OlaHsd substrain by electroporation and chimeric mice were generated. F1 mice were generated by breeding with C57BL/6 females. F2 homozygous mutant mice were produced by intercrossing F1 heterozygous males and females." According to Hasty et al., introduction of such a large piece of exogenous DNA into the gene would be expected to result in a null allele.

The Examiner has not presented any evidence supporting her position that the claimed mouse, a mouse having a null protease-like allele, was not made. Applicant reminds the Examiner that a specification is presumed to be truthful.

With regard to predictability, the Applicant again points out that enablement is evaluated with respect to the claimed invention: a transgenic mouse having a null protease-like allele. The claim encompasses two possibilities: a mouse having a single null allele (heterozygous) and a mouse having two null alleles (homozygous). The specification clearly sets forth how to make and use the mice. The heterozygous mice are useful for breeding homozygous mice and for gene expressions analyses, as well as for phenotypic evaluation. The specification and Examples show how to use the mice in phenotypic analyses to determine the function of the gene. Any phenotypes associated with the heterozygous and homozygous null mice are inherent to the mice. Many of the phenotypes will not be associated with genotype and therefore will be the same as a wild-type mouse. Thus, the claimed mice will have "wild-type" phenotypes.

In addition, predicting phenotypes *a priori* must be distinguished from reproducibility of the phenotype of the claimed mouse. The general rule is that disruption of the coding sequence by a positive selection marker, as taught in the specification, will result in a null allele, which by definition involves ablation of gene function (see, for example, Hasty et al., *Gene Targeting, Principles, and Practice in Mammalian Cells* in Gene Targeting: *A Practical Approach*, ed.

Joyner, Oxford University Press 2000, p. 5). Ablation of function is expected to result in the same phenotypic response. The Examiner has not provided any support for the assertion that mice produced by the methods disclosed in the specification would not lead to a consistent phenotype.

Applicant submits the claimed invention is fully enabled. Withdrawal of the rejections is respectfully requested.

Rejection under 35 U.S.C. § 112, second paragraph

Claims 36-48 stand rejected on grounds of indefiniteness. The Examiner argues that the specification fails to describe "a null protease-like allele."

According to the Federal Circuit, satisfaction of the written description requirement is measured by the understanding of the ordinarily skilled artisan. The description must clearly allow persons of ordinary skill in the art to recognize that the inventor invented what is claimed (*Amgen v. Hoechst Marion Rousel* (65 USPQ2d 1385 (Fed. Cir. 2003)).

The written description is evaluated with respect to the claimed invention. In this case, what is claimed is a transgenic mouse with a null limulus clotting factor protease-like allele. According to the specification, a "'limulus clotting factor protease-like gene' refers to a sequence comprising SEQ ID NO:1 or comprising the sequence identified in Genebank as Accession No. AA833210; GI:2906938." (specification, page 7). One skilled in the art would clearly recognize that Applicant invented a mouse having a null protease-like allele.

The Examiner cites *Fiers v Revel* and *Amgen v Chugai.*

Both cases are clearly distinguishable from the present case. Both involved patents which claimed DNA sequences encoding certain proteins. Both cases involved the issue of when the applicant was in possession of the claimed nucleotide sequence.

In the present case, Applicant is not claiming a DNA sequence. Moreover, the mouse protease-like gene sequence is disclosed in the specification. Applicant submits that neither case cited by the Examiner is relevant to the present situation.

In re Shokal is also readily distinguishable. The case dealt with a prior art rejection and whether a prior application supported the claims of the application at issue. The court held that "[u]nder the circumstances noted we are of the opinion that neither the broad language relied on by appellants nor the specific examples given by them sufficient to identify or point out the

particular genus recited in either of the appealed claims, and that appellants' prior application and the affidavit submitted by them under Rule 131 do not, singly or collectively, constitute a sufficient disclosure to antedate Rothrock's filing date, so far as those claims are concerned." (Shokal at 286-7). Thus, the case is not relevant to the present situation, as there is no pending parent application whose disclosure has been questioned.

The Examiner cites *Fiddes v Baird* for the proposition that claims directed to mammalian FGF's were found to be unpatentable due to lack of written description.

Fiddes is likewise clearly distinguishable. In *Fiddes*, applicant Baird was claiming mammalian FGF although their specification disclosed a single species, a bovine pituitary FGF. Baird was not even in possession of the natural gene encoding bovine pituitary FGF.

In the present case, Applicant is not claiming mammalian protease-like gene, nor is Applicant even claiming mouse protease-like gene. Applicant is claiming a mouse having a null mouse lumulus clotting factor protease-like allele, where the endogenous gene is defined as a "sequence comprising SEQ ID NO:1 or comprising the sequence identified in Genebank as Accession No. AA833210; GI:2906938." - a single species. Applicant submits that one skilled in the art would acknowledge that Applicant was in possession of the claimed invention.

With regard to "protease-like allele," according to the specification, the "term "transgenic animal" refers to an animal that contains within its genome a specific gene that has been disrupted by the method of gene targeting. The transgenic animal includes both the heterozygote animal (*i.e.*, one defective allele and one wild-type allele) and the homozygous animal (*i.e.*, two defective alleles)." (page 7, lines 25-28).

According to Hasty (*Gene Targeting, Principles, and Practice in Mammalian Cells* in Joyner, Gene Targeting: *A Practical Approach*, Oxford University Press 2000) (copy of relevant pages attached),:

> Since the most common experimental strategy is to ablate the function of a target gene (null allele) by introducing a selectable marker gene . . .
>
> (page 1)(emphasis added).

According to Crawley (What's Wrong With My Mouse *Behavioral Phenotyping of Transgenic and Knockout Mice*, Wiley-Liss 2000) (copy of relevant pages attached):

> **Knockout mice** have a **gene deleted**. The **null mutant homozygous** knockout mouse is deficient in both alleles of a gene, the **heterozygote** is deficient in one of its two alleles

for the gene. The **genotype** is -/- for the null mutant, +/- for the heterozygote, and +/+ for the wildtype normal control.

(p. 2)(emphasis in original).

As noted above, there are two possibilities, a mouse having a single null protease-like allele (heterozygous mouse) or a mouse having two null protease-like alleles. The Applicant made both of these. One skilled in the art would clearly appreciate that the Applicant fully possessed the claimed invention, a transgenic mouse having a null protease-like allele.

With regard to "visible marker," the claims have been amended, thus addressing the Examiners' remarks.

Applicant submits that the claimed invention satisfies the written description requirement. Withdrawal of the rejections is respectfully requested.

Rejection under 35 U.S.C. § 112, second paragraph

Claims 36-48 are rejected as allegedly indefinite under 35 U.S.C. § 112, second paragraph.

The Examiner argues that "protease-like" is unclear as it is not understood how a gene can be protease-like.

Applicant submits that the term would be clearly understood by one skilled in the art. For example, a search of the NCBI website for "protease-like gene" reveals an article by Liu et al., entitled, "Identification of a novel serine protease-like gene, the expression of which is down-regulated during breast cancer progression" (*Cancer Res.* 1996 Jul 15;56(14):3371-9)). Several others examples were also found. As the term would be understood by one skilled in the art, the claim satisfies the definiteness requirement.

With regard to a null allele comprising the sequence of SEQ ID NO:1; the null allele comprising exogenous DNA; and the term "capable of," the language has been deleted from the relevant claims, rendering the rejections moot.

Withdrawal is respectfully requested.

In view of the above amendments and remarks, Applicant respectfully requests a Notice of Allowance. If the Examiner believes a telephone conference would advance the prosecution

of this application, the Examiner is invited to telephone the undersigned at the below-listed telephone number.

The Commissioner is hereby authorized to charge any deficiency or credit any overpayment to Deposit Account No. **502775**.

Respectfully submitted,

7-19-05
Date

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APPENDIX

Spencer, NHGRI; Background on Mouse as a Model Organism

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URL of this page: http://www.genome.gov/10005834

Background on Mouse as a Model Organism

Over the past century, the mouse has developed into the premier mammalian model system for genetic research. Scientists from a wide range of biomedical fields have gravitated to the mouse because of its close genetic and physiological similarities to humans, as well as the ease with which its genome can be manipulated and analyzed.

Although yeasts, worms and flies are excellent models for studying the cell cycle and many developmental processes, mice are far better tools for probing the immune, endocrine, nervous, cardiovascular, skeletal and other complex physiological systems that mammals share. Like humans and many other mammals, mice naturally develop diseases that affect these systems, including cancer, atherosclerosis, hypertension, diabetes, osteoporosis and glaucoma. In addition, certain diseases that afflict humans but normally do not strike mice, such as cystic fibrosis and Alzheimer's, can be induced by manipulating the mouse genome and environment. Adding to the mouse's appeal as a model for biomedical research is the animal's relatively low cost of maintenance and its ability to quickly multiply, reproducing as often as every nine weeks.

Mouse models currently available for genetic research include thousands of unique inbred strains and genetically engineered mutants. There are mice prone to different cancers, diabetes, obesity, blindness, Lou Gehrig's disease, Huntington's disease, anxiety, aggressive behavior, alcoholism and even drug addiction. Immunodeficient mice can also be used as hosts to grow both normal and diseased human tissue, a boon for cancer and AIDS research.

In the early days of biomedical research, scientists developed mouse models by selecting and breeding mice to produce offspring with the desired traits. Researchers also learned to produce useful, new models of genetic disease quickly and in large numbers by exposing mice to DNA-damaging chemicals, a process known as chemical mutagenesis.

In recent decades, researchers have utilized an array of innovative genetic technologies to produce custom-made mouse models for a wide array of specific diseases, as well as to study the function of targeted genes. One of the most important advances has been the ability to create transgenic mice, in which a new gene is inserted into the animal's germline. Even more powerful approaches, dependent on homologous recombination, have permitted the development of tools to "knock out" genes, which involves replacing existing genes with altered versions; or to "knock in" genes, which involves altering a mouse gene in its natural location. To preserve these extremely valuable strains of mice and to assist in the propagation of strains with poor reproduction, researchers have taken advantage of state-of-the-art reproductive technologies, including cryopreservation of embryos, *in vitro* fertilization and ovary transplantation.

The Jackson Laboratory, a publicly supported national repository for mouse models in Bar Harbor, Maine, has played a crucial role in the development of the mouse into the leading model for biomedical research. Established in 1929, the non-profit center pioneered the use of inbred laboratory mice to uncover the genetic basis of human development and disease. In fact, the famous "Black 6" or C57BL/6J mouse strain whose genome is the focus of the landmark sequencing effort was developed in the early 1920s by The Jackson Laboratory founder Clarence Cook Little.

Today, researchers at The Jackson Laboratory pursue projects in areas that include cancer, development and aging, immune system and blood disorders, neurological and sensory disorders, and metabolic diseases. Informatics researchers work with the public sequencing consortium to curate and integrate the sequenced mouse genome data with the wealth of biological knowledge collected in Jackson's Mouse Genome Informatics resource.

In addition, The Jackson Laboratory distributes 2,700 different strains and stocks as breeding mice, frozen embryos or DNA samples. In FY 2002 alone, the lab supplied approximately 2 million mice to the international scientific community.

Listed below is a sampling of mouse models developed and/or distributed by The Jackson Laboratory, along with brief descriptions of the human diseases they are helping scientists to understand:

- Down Syndrome - One of the most common genetic birth defects in humans, occurring once in every 800 to 1,000 live births, Down syndrome results from an extra copy of chromosome 21, an abnormality known as trisomy. The Ts65Dn mouse, developed at The Jackson Laboratory, mimics trisomy 21 and exhibits many of the behavioral, learning, and physiological defects associated with the syndrome in humans, including mental deficits, small size, obesity, hydrocephalus and thymic defects. This model represents the latest and best improvement of Down syndrome models to facilitate research into the human condition.
- Cystic Fibrosis (CF) - The *Cftr* knockout mouse has helped advance research into cystic fibrosis, the most common fatal genetic disease in the United States today, occurring in approximately one of every 3,300 live births. Scientists now know that CF is caused by a small defect in the gene that manufactures CFTR, a protein that regulates the passage of salts and water in and out of cells. Studies with the *Cftr* knockout have shown that the disease results from a failure to clear certain bacteria from the lung, which leads to mucus retention and subsequent lung disease. These mice have become models for developing new approaches to correct the CF defect and cure the disease.
- Cancer - The p53 knockout mouse has a disabled *Trp53* tumor suppressor gene that makes it highly susceptible to various cancers, including lymphomas and osteosarcomas. The mouse has emerged as an important model for human Li-Fraumeni syndrome, a form of familial breast cancer.
- Glaucoma - The DBA/2J mouse exhibits many of the symptoms that are often associated with human glaucoma, including elevated intraocular pressure. Glaucoma is a debilitating eye disease that is the second leading cause of blindness in the United States.
- Type 1 Diabetes - This autoimmune disease, also known as Juvenile Diabetes, or Insulin Dependent Diabetes Mellitus (IDDM), accounts for up to 10 percent of diabetes cases. Non-obese Diabetic (NOD) mice are enabling researchers to identify IDDM susceptibility genes and disease mechanisms.
- Type 2 Diabetes - A metabolic disorder also called Non-Insulin Dependent Diabetes Mellitus (NIDDM), this is the most common form of diabetes and occurs primarily after age 40. The leading mouse models for NIDDM and obesity research were all developed at The Jackson Laboratory: Cpe^{fat}, Lep^{ob}, $Lepr^{db}$ and *tub*.
- Epilepsy - The "slow-wave epilepsy," or *swe,* mouse is the only model to exhibit both of the two major forms of epilepsy: petit mal (absence) and grand mal (convulsive). It shows particular promise for research into absence seizures, which occur most often in children.
- Heart Disease - Elevated blood cholesterol levels and plaque buildup in arteries within three months of birth (even on a low-fat diet) are characteristics of several experimental models for

human atherosclerosis: the *Apoe* knockout mouse and C57BL/6J.

- Muscular Dystrophy - The *Dmd* ^{mdx} mouse is a model for Duchenne Muscular Dystrophy, a rare neuromuscular disorder in young males that is inherited as an X-linked recessive trait and results in progressive muscle degeneration.
- Ovarian Tumors - The SWR and SWXJ mouse models provide excellent research platforms for studying the genetic basis of ovarian granulosa cell tumors, a common and very serious form of malignant ovarian tumor in young girls and post-menopausal women.

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Last Reviewed: September 2004

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The Knockout Mouse Project

Mouse knockout technology provides a powerful means of elucidating gene function *in vivo*, and a publicly available genome-wide collection of mouse knockouts would be significantly enabling for biomedical discovery. To date, published knockouts exist for only about 10% of mouse genes. Furthermore, many of these are limited in utility because they have not been made or phenotyped in standardized ways, and many are not freely available to researchers. It is time to harness new technologies and efficiencies of production to mount a high-throughput international effort to produce and phenotype knockouts for all mouse genes, and place these resources into the public domain.

Now that the human and mouse genome sequences are known[1–3], attention has turned to elucidating gene function and identifying gene products that might have therapeutic value. The laboratory mouse (*Mus musculus*) has had a prominent role in the study of human disease mechanisms throughout the rich, 100-year history of classical mouse genetics, exemplified by the lessons learned from naturally occurring mutants such as agouti[4], reeler[5] and obese[6]. The large-scale production and analysis of induced genetic mutations in worms, flies, zebrafish and mice have greatly accelerated the understanding of gene function in these organisms. Among the model organisms, the mouse offers particular advantages for the study of human biology and disease: (i) the mouse is a mammal, and its development, body plan, physiology, behavior and diseases have much in common with those of humans; (ii) almost all (99%) mouse genes have homologs in humans; and (iii) the mouse genome supports targeted mutagenesis in specific genes by homologous recombination in embryonic stem (ES) cells, allowing genes to be altered efficiently and precisely.

The ability to disrupt, or knock out, a specific gene in ES cells and mice was developed in the late 1980s (ref. 7), and the use of knockout mice has led to many insights into human biology and disease[8–11]. Current technology also permits insertion of 'reporter' genes into the knocked-out gene, which can then be used to determine the temporal and spatial expression pattern of the knocked-out gene in mouse tissues. Such marking of cells by a reporter gene facilitates the identification of new cell types according to their gene expression patterns and allows further characterization of marked tissues and single cells.

Appreciation of the power of mouse genetics to inform the study of mammalian physiology and disease, coupled with the advent of the mouse genome sequence and the ease of producing mutated alleles, has catalyzed public and private sector initiatives to produce mouse mutants on a large scale, with the goal of eventually knocking out a substantial portion of the mouse genome[12,13]. Large-scale, publicly funded gene-trap programs have been initiated in several countries, with the International Gene Trap Consortium coordinating certain efforts and resources[14–17].

Despite these efforts, the total number of knockout mice described in the literature is relatively modest, corresponding to only ~10% of the ~25,000 mouse genes. The curated Mouse Knockout & Mutation Database lists 2,669 unique genes (C. Rathbone, personal communication), the curated Mouse Genome Database lists 2,847 unique genes, and an analysis at Lexicon Genetics identified 2,492 unique genes (B.Z., unpublished data). Most of these knockouts are not readily available to scientists who may want to use them in their research; for example, only 415 unique genes are represented as targeted mutations in the Jackson Laboratory's Induced Mutant Resource database (S. Rockwood, personal communication).

The converging interests of multiple members of the genomics community led to a meeting to discuss the advisability and feasibility of a dedicated project to produce knockout alleles for all mouse genes and place them into the public domain. The meeting took place from 30 September to 1 October 2003 at the Banbury Conference Center at Cold Spring Harbor Laboratory. The attendees of the meeting are the authors of this paper.

Is a systematic project warranted?

A coordinated project to systematically knock out all mouse genes is likely to be of enormous benefit to the research community, given the demonstrated power of knockout mice to elucidate gene function, the frequency of unpredicted phenotypes in knockout mice, the potential economies of scale in an organized and carefully planned project, and the high cost and lack of availability of knockout mice being made in current efforts. Moreover, implementing such a systematic and comprehensive plan will greatly accelerate the translation of genome sequences into biological insights. Knockout ES cells and mice currently available from the public and private sectors should be incorporated into the genome-wide initiative as much as possible, although some may be need to be produced again if they were made with suboptimal methods (e.g., not including a marker) or if their use is restricted by intellectual property or other constraints. The advantages of such a systematic and coordinated effort include efficient production with reduced costs; uniform use of knockout methods, allowing for more comparability between knockout mice; and ready access to mice, their derivatives and data to all researchers without encumbrance. Solutions to the logistical, organizational and informatics issues associated with producing, characterizing and distributing such a large number of

*The Comprehensive Knockout Mouse Project Consortium**

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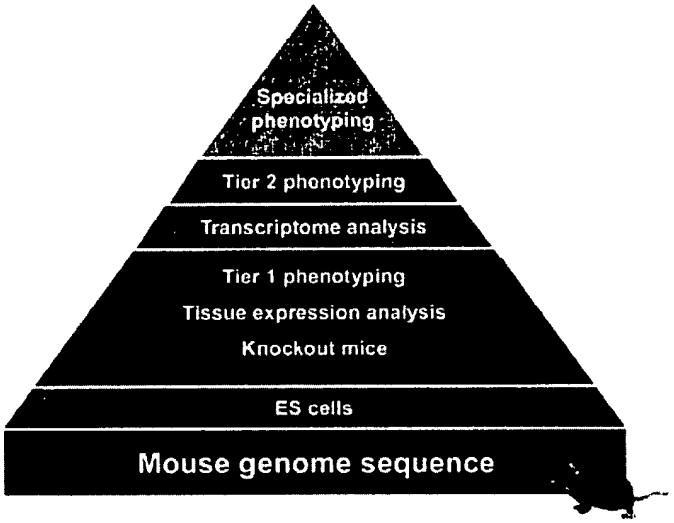

Figure 1 Structure of resource production in the proposed KOMP. Using the mouse genome sequence as a foundation, knockout alleles in ES cells will be produced for all genes. A subset of ES cell knockouts will be used each year to produce knockout mice, determine the expression pattern of the targeted gene in a variety of tissues and carry out screening-level (Tier 1) phenotyping. In a subset of mouse lines, transcriptome analysis and more detailed system-specific (Tier 2) phenotyping will be done. Finally, specialized phenotyping will be done on a smaller number of mouse lines with particularly interesting phenotypes. All stages will occur within the purview of the KOMP except for the specialized phenotyping, which will occur in individual laboratories with particular expertise.

mice will draw from the experience of related projects in the private sector and in academia, which have made or phenotyped hundreds of knockout mice using a variety of techniques. Lessons learned from these projects include the need for redundancy at each step to mitigate pipeline bottlenecks and the need for robust informatics systems to track the production, analysis, maintenance and distribution of thousands of targeting constructs, ES cells and mice.

Null-reporter alleles should be created

The project should generate alleles that are as uniform as possible, to allow efficient production and comparison of mouse phenotypes. The alleles should achieve a balance of utility, flexibility, throughput and cost. A null allele is an indispensable starting point for studying the function of every gene. Inserting a reporter gene (*e.g.*, β-galactosidase or green fluorescent protein) allows a rapid assessment of which cell types normally support the expression of that gene. Therefore, we propose to produce a null-reporter allele for each gene. Making each mutation conditional in nature by adding *cis*-elements (*e.g.*, *loxP* or FRT sites) would be desirable, but we do not advocate this as part of the mutagenesis strategy unless the technological limitations currently associated with generating conditional targeted mutations on a large scale and in a cost-effective manner can be overcome.

A combination of methods should be used

Various methods can be used to create mutated alleles, including gene targeting, gene trapping and RNA interference. Advantages of conventional gene targeting include flexibility in design of alleles, lack of limitation to integration hot spots, reliability for producing complete loss-of-function alleles, ability to produce reporter knock-ins and conditional alleles, and ability to target splice variants and alternative promoters. BAC-based targeting has the potential advantages of higher recombination efficiencies and flexibility for producing complex mutated alleles[18]. Gene trapping is rapid, is cost-effective and produces a large variety of insertional mutations throughout the genome but can be somewhat less flexible[17,19–21]. There is uncertainty regarding the percentage of gene traps that produce a true null allele and the fraction of the genome that can ultimately be covered by gene-trap mutations. Trapping is not entirely random but shows preference for larger transcription units and genes more highly expressed in ES cells. In recent studies, gene trapping was estimated to potentially produce null alleles for 50–60% of all genes, perhaps more if a variety of gene-trap vectors with different insertion characteristics is used[17,21]. RNA interference offers enormous promise for analysis of gene function in mice[22] but is not yet sufficiently developed for large-scale production of gene modifications capable of reliably producing true null alleles. Both gene-targeting and gene-trapping methods are suitable for producing large numbers of knockout alleles, and, given their complementary advantages, a combination of these methods should be used to produce the genome-wide collection of null-reporter alleles most efficiently.

What should the deliverables be?

A genome-wide knockout mouse project could deliver to the research community a trove of valuable reagents and data, including targeting and trapping constructs and vectors, mutant ES cell lines, live mice, frozen sperm, frozen embryos, phenotypic data at a variety of levels and detail, and a database with data visualization and mining tools. At a minimum, we believe that a comprehensive genome-wide resource of mutant ES cell lines from an inbred strain, each with a different gene knocked out, should be produced and made available to the community. Choosing an inbred line (129/SvEvTac or C57BL/6J), and evaluating the alternative of using F_1 ES cells and tetraploid aggregation to provide potential time savings, merits additional scientific review and discussion[23,24]. ES cells should be converted into mice at a rate consistent with project funding and the ability of the worldwide scientific community to analyze them. Although the value and cost-effectiveness of systematically characterizing the mice is a matter of debate, a limited set of broad and cost-effective screens, probably including assessment of developmental lethality, physical examination, basic blood tests, and histochemical analysis of reporter gene expression, would be useful. More detailed phenotyping, based on findings from the initial screen or existing knowledge of the gene's function, could be done at specialized centers. All ES cell clones and mice (as frozen embryos or sperm) should be available to any researcher at minimal cost, and all mouse phenotyping and reporter expression data should be deposited into a public database.

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In determining how to implement the project, utility to the research community should be the standard for judging value. Each step after ES cell generation (*e.g.*, mouse creation, breeding, expression analysis, phenotyping) will make the resource useful to more researchers but will also increase costs and scientific complexity. We therefore advocate a 'pyramid' structure for the project (Fig. 1). At the base of the pyramid is the genome-wide collection of mutant ES cells for every mouse gene. Over time, a subset of these mutant ES cells should be made into mice and characterized with an initial phenotype screen (Tier 1; Fig. 1) and analysis of tissue reporter-gene expression. A subset of these lines should be profiled by microarray analysis, and a subset of these profiled by system-specific (Tier 2) phenotyping, based on the results of the Tier 1 phenotyping, array studies, existing knowledge of the gene's function and the gene's tissue expression pattern. With time, the upper tiers of the pyramid will be filled out, eventually transforming the pyramid into a cube, with information of all types available for all genes.

This project will require the resolution of numerous intellectual property claims involving the production and use of knockout mice. To deal with the existing patents that cover the technologies and processes involved in the production of mutant mice, we suggest that a 'patent pool', such as that used in the semiconductor industry[25], should be generated. Several individuals who represent entities that control patents on mouse knockout technologies are authors on this paper, and they agree with this approach. We also agree that any mutant ES cells or mice produced should be placed immediately in the public domain.

Mechanisms and costs

ES cell production. Automated knockout construct and ES cell production should be carried out in coordinated centers to ensure efficiency and uniformity. We estimate that most known mouse genes could be knocked out in ES cells within 5 years, using a combination of gene-trapping and gene-targeting techniques. Gene trapping can produce a large number of mutated alleles quickly, but its progress should be monitored closely to determine when its yield of new genes diminishes[17] and, therefore, when targeting should be increasingly relied on. As large-scale trapping projects have already defined gene classes that probably cannot be knocked out by trapping (*e.g.*, single-exon GPCRs, genes that are not expressed in ES cells), we propose that targeting begin on those classes immediately. All ES cells should be made available to the research community, because this collection itself would be a valuable resource. Efforts in the public and private sectors have already knocked out many genes in ES cells, and, to the degree that the alleles produced fit the prescribed characteristics (*i.e.*, null alleles with a reporter) and are available, every effort should be made to incorporate these into the planned public resource. Costs for generating this part of the resource were estimated at between $9–11 million/year for five years (these and all subsequent figures are direct costs).

Mouse production. The subset of ES cells made into mice each year should be chosen by a peer-review process. Central facilities for high-efficiency mouse production, genotyping, breeding, maintenance and archiving should be funded, to take advantage of efficiencies of scale in mouse creation and distribution. Researchers could apply to produce groups of mice outside the centers, as long as they meet the cost specifications of the program. All mice should be made available immediately to researchers as frozen embryos or sperm, for nominal distribution cost. An initial target of 500 new mouse lines per year would double the current rate at which new genes are knocked out in the public sector; we feel that this rate is within the capacity of the biomedical research community worldwide to absorb and analyze. We estimated the initial cost of this level of mouse production to be $12.5–15 million per year.

Reporter tissue expression analysis. Approximately 30 tissues from adult and developmental stages should be sampled to cover the main organ systems. Analysis methods should be customized to the organ system and marker, and a searchable database of the sites of gene expression, and the images showing them, should be produced. Centers to carry out these analyses and data curation should be selected by peer review. We estimated the cost of this component for 500 mouse lines to be $2.5–5 million per year, depending on how much tissue sectioning and cell-level analysis is done.

Phenotyping. Tier 1 phenotyping should be a low-cost screen for clear phenotypes and should be done on all mouse lines produced. Tier 1 should include home-cage observation, physical examination, blood hematological and chemistry profiles, and skeletal radiographs. The centers producing the mice should carry out the Tier 1 analyses, at an estimated cost of $2.5 million per year for 500 lines. Selected lines, chosen on the basis of findings from Tier 1 phenotyping, tissue expression patterns, microarray data and the scientific literature, should undergo more detailed and system-focused Tier 2 phenotyping. Tier 2 phenotyping should be done in specialized phenotyping centers, akin to those already in operation for phenotyping of mice produced by ENU mutagenesis. All Tier 1 and Tier 2 phenotyping should be done on a uniform genetic background by dedicated groups of individuals in single locations, to facilitate consistency and cross-comparison of results among different mouse lines. All Tier 1 and Tier 2 phenotyping results should be deposited into a central project database freely accessible to the research community. More detailed and specialized phenotyping could be done by individual researchers in their own laboratories; deposition of this more detailed phenotype data would be encouraged.

Transcriptome analysis. Transcriptome profiling of tissues from each knockout line, collected in a uniform way across all mice and tissues and placed into a searchable relational database, would add substantially to the scientific value of the project, though it would also add considerably to its cost. Transcriptome analysis should therefore be done on a subset of mice, chosen by peer review. We estimate that, with the best currently available array technology, an analysis of ten tissues would cost ~$18,000 per line.

Conclusions

This project, tentatively named the Knockout Mouse Project (KOMP), will be a crucial step in harnessing the power of the genome to drive biomedical discovery. By creating a publicly available resource of knockout mice and phenotypic data, KOMP will knock down barriers for biologists to use mouse genetics in their research. The scientific consensus that we achieved—that a dedicated project should be undertaken to produce mutant mice for all genes and place them into the public domain—is important but is only the beginning. Implementation of these recommendations will require additional input from the greater scientific community, including those responsible for programmatic direction and financial support of biomedical research in the public and private sectors. This ambitious and historic initiative must be carried out as a collaborative effort of the worldwide scientific community, so that all can contribute their skills, and all can benefit. International discussions among scientific and programmatic staffs since the Banbury meeting at Cold Spring Harbor, in both the public and private sectors, have shown that there is great enthusiasm and commitment to this vision. The next step for KOMP will be to move this visionary plan from conceptualization to implementation, with an urgency befitting the benefits it will bring to science and medicine.

URLs. The curated Mouse Knockout & Mutation Database is available at http://research.bmn.com/mkmd/. The curated Mouse Genome Database is available at http://www.informatics.jax.org/. *Patent pools: A solution to the problem of access in biotechnology patents?* is available at http://www.uspto.gov/web/offices/pac/dapp/opla/patentpool.pdf.

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Laboratory Animal Science
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Vol 49, No 2
April 1999

Special Topic Overview

Interpretation of Phenotype in Genetically Engineered Mice

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Background and Purpose: **In mice, genetic engineering involves two general approaches—addition of an exogenous gene, resulting in transgenic mice, and use of knockout mice, which have a targeted mutation of an endogenous gene. The advantages of these approaches is that questions can be asked about the function of a particular gene in a living mammalian organism, taking into account interactions among cells, tissues, and organs under normal, disease, injury, and stress situations.**

Methods: **Review of the literature concentrating principally on knockout mice and questions of unexpected phenotypes, lack of phenotype, redundancy, and effect of genetic background on phenotype will be discussed.**

Conclusion: **There is little gene redundancy in mammals; knockout phenotypes exist even if none are immediately apparent; and investigating phenotypes in colonies of mixed genetic background may reveal not only more phenotypes, but also may lead to better understanding of the molecular or cellular mechanism underlying the phenotype and to discovery of modifier gene(s).**

One often hears the comment that genetically engineered mice, especially knockout mice, are not useful because they frequently do not yield the expected phenotype, or they don't seem to have any phenotype. These expectations are often based on years of work, and in some instances, thousands of publications of mostly in vitro studies. Examples of unexpected phenotypes, based largely on experience with transforming growth factor beta (*Tgfb*) and basic fibroblast growth factor (*Fgf2*) knockout and transgenic mice, will be presented to discuss possible reasons for unexpected knockout phenotypes. The conclusions will be that the knockout phenotypes do, in fact, provide accurate information concerning gene function, that we should let the unexpected phenotypes lead us to the specific cell, tissue, organ culture, and whole animal experiments that are relevant to the function of the genes in question, and that the absence of phenotype indicates that we have not discovered where or how to look for a phenotype.

Before entering into how one should interpret unexpected knockout phenotypes and how one should deal with lack of knockout phenotypes, it is necessary to give a brief introduction into how knockout mice are made. For detailed information, the following reviews are suggested (1–4). Transgenic technology has had a long history; thus, an introduction to that technology will not be given here. Rather, the following reviews are suggested (5, 6). At this juncture, it should be noted that, although transgenic vertebrates ranging from fish to bovids have been produced, knockout technology has to date been successful only in mice, even though embryonic stem (ES) cells have been produced from several other species, including hamster (7), rat (8), rabbit (9, 10), pig (11–13), bovine (14, 15), and zebrafish (16). Consequently, the entire discussion will be focused on mice.

Knockout mice are generated by the injection of genetically engineered or gene-targeted ES cells into a mouse blastocyst to generate a chimeric embryo, which in turn can pass on the engineered gene to its offspring. ES cell lines are established from the inner cell mass of a mouse blastocyst, so that when injected into blastocysts, the ES cells can incorporate into the inner cell mass of the recipient blastocysts thereby chimerizing them. Subsequent to transfer of the chimeric blastocysts into uteri of pseudopregnant mice, chimeric mice are born. If the germline of a chimeric mouse is colonized by cells derived from the injected ES cells, the chimera is termed a "germline" chimera. Some of the offspring of the germline chimeras will then carry the engineered gene in their genomes. Gene targeting in ES cells uses the ES cells' DNA repair apparatus to bring about homologous recombination between an exogenous DNA fragment transfected into the ES cell and its homologous region in the genome. Homologous recombination usually results in replacement of the endogenous region with the exogenous fragment, thereby altering the endogenous gene in a prespecified manner. There are many variations on this procedure by which genes can be altered not only to ablate function, but also to make more subtle mutations (17–19). Such procedures can be used to introduce point mutations, remove specific splicing products, switch isoforms, and humanize genes. In addition, technology has recently been

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developed to make conditional and inducible knockouts in which gene function is ablated either in a developmentally specified tissue (20–22) or in an inducible manner (23–26). These techniques, though exciting, will not be further discussed.

Extensive nonredundancy in the TGFβ family: Several thousand cell culture studies on the three mammalian transforming growth factor beta proteins (TGFβs 1, 2, and 3) have implicated these growth and differentiation factors in the function of nearly every cell type studied. Expression studies indicated unique and overlapping expression of the three TGFβs (27, 28). For example, overlapping protein localization was found in all gut epithelia, all layers of the skin, all three muscle types, kidney tubules, lung bronchi, cartilage, and bone (Table 1). Together with the fact that all three TGFβs signal through a common TGF type-II receptor (Figure 1), these data strongly suggest considerable redundancy in function. Consequently, it is surprising that, of the >30 phenotypes of the three *Tgfb* knockout mice that we have described (29–31), none appear to be overlapping (Table 2). These results indicate extensive nonredundancy between TGFβ ligands even though there is considerable overlap in expression. Of course, these results do not rule out the possibility of some redundancy in some tissues. Combination of the ligand knockouts would uncover such situations, and it is likely that a few will exist, but 30 nonoverlapping phenotypes for three ligands strongly suggests that a vast number of their functions are not redundant.

There are several possible explanations for how there can be so much overlap in ligand expression and yet so much specific ligand function. First, TGFβs are secreted as latent peptides and must be activated before they can bind receptors (32–35). The mechanism by which this extracellular processing occurs is not well understood and may be different for each TGFβ. Hence, ligand processing presumably determines some functional specificity for the three TGFβs. Second, there is a third type of TGFβ receptor, TGFβR3, that can interact with ligand and receptor types I and II before cytoplasmic signaling can occur, though involvement of TGFβR3 is not essential for signaling (36–38). Association with type III receptors is thought to enhance some TGFβR1 and 2/ligand interactions. Upon ligand binding, the serine/threonine receptor TGFβR2 then associates with and phosphorylates the transmembrane serine/threonine receptor TGFβR1, which in turn initiates a phosphorylation-mediated signaling cascade. Hence, combinatorial receptor/ligand interactions will also determine functional specificity. Third, signaling from TGFβR1 can occur through two cytoplasmic signaling proteins called SMAD2 and 3 (39, 40) and, perhaps, through a third called SMAD5 (41). In addition, SMAD6 and 7 can also interact with the other SMADs to inhibit signaling (42–44). Hence, differential SMAD protein interactions with transcriptional machinery will probably also determine functional specificity for the three TGFβ ligands. Finally, there may be several non-transcriptional signaling pathways for TGFβs. For example, we have found that TGFβ1-deficient platelets from *Tgfb1* knockout mice have impaired platelet aggregation that can be restored by incubating isolated platelets with recombinant TGFβ1 (unpublished observations). Because platelets do not have a nucleus, there must exist a signaling pathway that is nontranscriptional. In summary, given the complexities of ligand processing, receptor interactions, and signaling pathways, it becomes clear why redundancy in TGF1, 2, and 3 function has not been detected at the whole animal level, even though there is considerable overlap in expression of *Tgfb* gene family members. Consequently, if other gene families function with similar complexity, it is likely that, in the final analysis, little functional redundancy will be found within gene families.

Two striking examples of apparent functional redundancy are worth considering. The first involves myogenic genes, and the second involves retinoic acid receptors. Contrary to early interpretations, redundancy does not now appear to be

Table 1. Protein expression of transforming growth factor beta (TGFβ) 1, 2, and 3

Tissue/cell type	TGFβ1	TGFβ2	TGFβ3
Cartilage			
Perichondrium	+++	+	++
Chondrocytes	+	++	++
Bone			
Periosteum	++	-	+
Osteocytes		++	++ ++
Tooth			
Ameloblasts	+	-	+
Odontoblasts	-	++	-
Pulp	+	+++	+
Muscle			
Smooth	+	+	++
Cardiac	+	+	+++
Skeletal	+	++	
Lung			
Bronchi	++	++	++
Alveoli	-	-	-
Blood vessels			
Endothelium	-	-	++
Smooth muscle	+	+	+++
Kidney			
Tubules	++	++	++
Basement membrane	-	+++	-
Adrenal			
Cortex	+++	+++	-
Medulla	-	-	-
Gut			
Esophageal epithelium	+++	+	+
Gastric epithelium	+++	+	+
Intestinal epithelium	++	+	+
Basement membrane	-	+++	-
Muscularis	+	+	++
Liver			
Capsule	-	-	++
Parenchyma	-	-	-
Megakaryocytes	+	-	++
Eye			
Lens epithelium	-	-	-
Lens fibers	+++	+	+
Ear			
Cochlear epithelium	-	+	+++
Basement membrane	-	+++	-
CNS			
Meninges	+	+++	+
Glia	-	++	++
Choroid plexus	-	-	++
Skin			
Periderm	++	+	++
Epidermis	+++	+++	+++
Dermis	+	+++	+
Hair follicles	++	++	+

The polyclonal antibodies used were specific for residues 4–19 of TGFβ1 and 2 and residues 9–20 of TGFβ3. The avidin-biotin system was used for staining. Data obtained from immunohistochemical study of Pelton et al. (28). Reproduced from *The Journal of Cell Biology*, 1991,115:1091–1105, by copyright permission of The Rockefeller University Press.

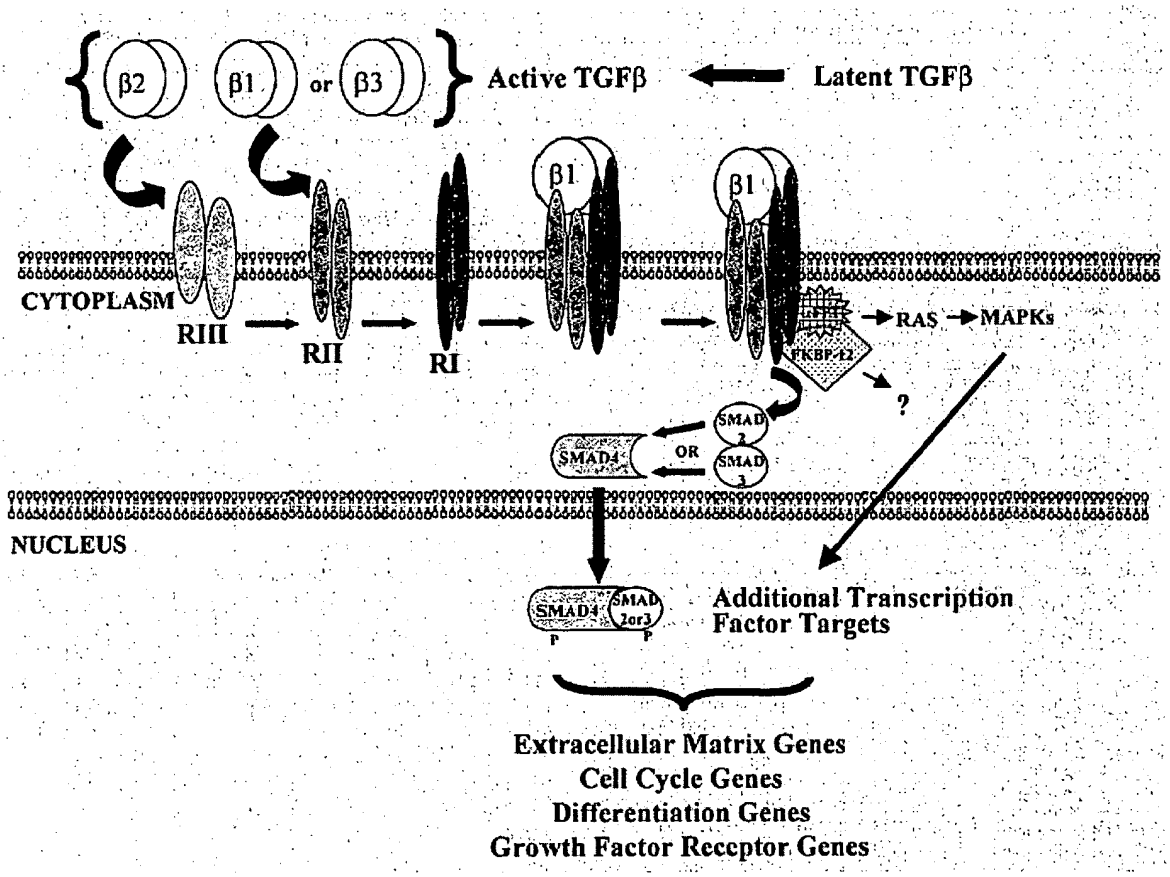

Figure 1. TGFβ signaling pathway. The TGFβ ligands, TGFβ1 (β1), TGFβ2 (β2), and TGFβ3 (β3), exist primarily in a latent form in vivo and are activated by mechanisms not yet clear. In general, TGFβ2 interacts with a TGFβ type III receptor (RIII) before interaction with TGFβ type II (RII) and TGFβ type I (RI) receptors; whereas, the TGFβ1 and TGFβ3 ligands can interact directly with the type II receptor. The ligand receptor complexes can then associate with several cytoplasmic molecules, farnesyl protein transferase (FPT) and FK506 binding protein-12 (FKBP-12), being two potential examples. The receptor-ligand complex signals to the nucleus through threonine/serine phosphorylation of a series of SMAD proteins (related to the Drosophila "mothers against decapentaplegic" protein) which then elicit transciptional regulation of extacellular matrix, cell cycle, differentiation and growth factor receptor genes. The roles of the associated cytoplasmic molecules FPT and FKBP-12 are not clear but are thought to involve RAS pathway signaling and modulation of signaling through the SMAD proteins.

the case for two of the myogenic genes known to be essential for specification of vertebrate skeletal muscle, *Myod* and *Myf5*. Even though the individual knockouts have muscle, and only the combined knockouts do not have muscle (45), it is now clear that each gene functions in the specification of distinct muscle cell lineages. Consequently, in the absence of one source of muscle cells, the other source may compensate for that (46, 47). This should be termed developmental compensation, rather than gene redundancy. On the other hand, with respect to retinoic acid receptors, there is also good evidence for functional redundancy. Similar to the myogenic genes, retinoic acid receptor gene knockout mice have few phenotypes, whereas the combined knockouts have many phenotypes (48, 49). Whether this turns out to be gene redundancy or another case of developmental compensation remains to be determined.

Lack of phenotype: As is the case for TGFβ, there also is a multitude of reports indicating that the FGFs 1 and 2 have important roles in numerous cell types and tissues. Consequently, when the *Fgf2* gene was knocked out by gene targeting, it was quite surprising that there was no obvious phenotype (50). The *Fgf2*$^{-/-}$ animals live a long, healthy life, and fertility and fecundity are normal. Even the pituitary gland, which is the best source of FGF2, appears not to have morphologic defects. The only evidence for any developmental abnormalities is found in hematopoiesis (50), where blood platelet counts are high, and in the cerebral cortex (51, 52), where morphometric analysis reveals decreased cell density. Clearly, these abnormalities are minor, compared with expectations. This was all the more evident because our transgenic mice, in which the human *FGF2* gene was ubiquitously overexpressed by the phosphoglycerate kinase pro-

Table 2. Nonoverlapping phenotypes of *Tgfb1*, *2*, and *3* knockout mice and the penetrance of those phenotypes

Knockout mouse phenotype	Penetrance (%)[a]
Tgfb1	
Embryo lethalities	50
Preimplantation lethality	50
Yolk sac lethality	50[b]
Adult phenotypes	50
Multifocal autoimmunity	100[c]
Platelet defect	100[c,d]
Colon cancer	100[c,d]
Failing heart	100[c,d]
Tgfb2 (all perinatal lethalities)	
Heart defects	100
Ventricular septum defects	94
Dual outlet right ventricle	19
Dual inlet left ventricle	25
Inner ear defect—lacks spiral limbus	100
Eyes	
Ocular hypercellularity	100
Reduced corneal stroma	100
Urogenital defects in kidney	
Dilated renal pelvis	30
Agenesis (females only)	20
Uterine horn ectopia	40
Testicular ectopia	100
Testis hypoplasia	20
Vas deferens dysgenesis	20
Lung-postnatal	
Dilated conducting airways	100
Collapsed bronchioles	100
Skeletal defects	
Occipital bone	100
Parietal bone	100
Squamous bone	100
Palatine bone (cleft palate)	22
Alisphenoid bone	100
Mandibular defects	100
Short radius and ulna	100
Missing deltoid tuberosity and third trochanter	94
Sternum malformations	25
Rib barreling	94
Rib fusions	13
Spina bifida	100
Tgfb3 (perinatal lethality)	
Cleft palate	100

[a] See Table 3 for background dependency of *Tgfb1* knockout phenotypes.
[b] Described in references 64, 67.
[c] Refers to percentage penetrance among animals that survive to birth.
[d] Unpublished observations.
Details on the remaining phenotypes can be found in the text and in references 29–31, 63.

moter (53), had very short legs, suggesting an important role of FGF2 in bone development, yet the bones of the knockout animals were normal. This apparent discrepancy between the transgenic and knockout mice indicates that some other FGF signals through the same FGF receptor as does FGF2, and that this other FGF is the true ligand that is important in bone development. Another possibility is that there is "developmental compensation" by alternative mechanisms. In other words, the absence of FGF2 may cause developmental abnormalities during bone development that are then compensated for by another developmental pathway. This alternative would not necessarily require a different FGF to be involved.

After we had made our first analysis of the *Fgf2* knockout mouse and did not find an obvious phenotype, it was easy to explain the "lack of phenotype" by invoking redundancy because there are at least 18 known *Fgf* genes. But in hindsight, it now appears more likely that all members of this large gene family have specific functions, even though they signal through receptors encoded by only four receptor genes (54). In *Fgf2* knockout mice, evidence was not found for upregulation of the two ligands most structurally related to FGF2, namely, FGFs 1 and 5 (50). Also, genetic combination of *Fgf2* and *Fgf5* (50) did not reveal redundancy between these similar genes. In addition, further analysis of the mice revealed roles being played in hematopoiesis and vascular tone control (50) as well as in brain development and wound healing (51, 52). Finally, in addition to *Fgf2*, *Fgfs* 3–5, 7, 8 also have been ablated by gene targeting, revealing functions in proliferation of the inner cell mass (*Fgf4*) (55); gastrulation and cardiac, craniofacial, forebrain, midbrain, and cerebellar development (*Fgf8*) (56); brain and inner ear development (*Fgf3*) (57, 58); and two aspects of hair development (*Fgf5* and 7) (59, 60). To date, comparison of *Fgf* knockout phenotypes from 6 of the 18 *Fgf* genes has not turned up overlap except possibly in the cerebellum. Together, these results indicate that each gene has important unique functions. Although a few redundant functions may eventually be found on combination of *Fgf2* with all other *Fgfs* except *Fgf5*, it is clear that 6 of the 18 *Fgf* genes studied by gene targeting have been associated with essentially unique knockout phenotypes.

To summarize, what originally appeared as "lack of phenotype" led many of us to the premature conclusion that other FGFs must have functions redundant to those of FGF2. However, further analysis of *Fgf2* knockout mice has since revealed a wealth of unique functions ranging from thrombocytosis and vascular tone control (50) to brain development and wound healing (51, 52). It is my expectation that further physiologic analysis of the *Fgf2* knockout mouse will reveal functions in the hypertrophic response to hypertension and responses to ischemia/reperfusion injury and bone injury. In the final analysis, it is likely that the major roles of FGF2 may have less to do with getting us to birth than with keeping us alive after birth, whereas several other FGFs clearly have developmental roles.

Effects of genetic background on phenotypic variation: From 100 years of mouse genetics, it has become clear that genetic background plays an important role in the susceptibility of mice to many disorders. Therefore, the phenotypes of knockout mouse strains will also have genetic background dependencies, as was first documented by the Magnuson and Wagner groups (61, 62). The *Tgfb1* knockout mice are an exceptional case in point (Table 3). On a mixed (50:50) 129 x CF1 background (CF1 is a partially outbred strain), about half of *Tgfb1* knockout mice die from a preimplantation developmental defect (63), and the other half die of an autoimmune-like multifocal inflammatory disease at about weaning age (29). If the targeted *Tgfb1* allele is backcrossed onto a C57BL/6 background, 99% of all knockout animals die of the preimplantation defect(63). However, if a *Tgfb1* knockout allele is put onto a mixed 129 x NIH/Ola x C57BL/6 background, embryo lethality is observed during yolk sac development, not during preimplantation development (64). With respect to the multifocal inflammatory disorder of *Tgfb1* knockout mice, if the targeted allele is put onto a 129 x CF1 mixed background (50:50), severe inflammation exists only in the stomach (29); on the mixed 129 x

Table 3. Background dependency of *Tgfb1* knockout phenotypes

Phenotype	Phenotype penetrance on various strains (%)						
	129 x CF1	129 x C57	129 x C3H	C57	129	C3H	129xC57x NIH/Ola
Preimplantation lethality	50	ND	ND	99	ND	ND	0
Yolk sac lethality[a]	0	0	ND	ND	ND	ND	50
Autoimmune disease	50	50	50	1	ND	ND	50
Gastric inflammation	90[b]	20[b]	ND	ND	ND	ND	ND
Intestinal inflammation	0	70[b]	ND	ND	ND	ND	ND
Colon cancer[c]	ND	ND	ND	ND	100	0	ND

Percentage of knockout animals of a given strain that have the designated phenotype.
[a]For details, see references 64, 67.
[b]Approximately 10% of animals with autoimmune disease have no detectable gastrointestinal tract inflammation.
[c]Unpublished observations.
ND = not determined.

NIH/Ola x C57BL/6 background, the intestines are more severely inflamed than is the stomach (65). Finally, on a predominantly 129 background (129 x CF1; ~97:3), *Tgfb1* knockout mice develop colon cancer if the inflammatory disorder can be eliminated by other genetic manipulations that render the mice immunodeficient (unpublished observations). However, on a predominantly C3H background, immunodeficient *Tgfb1* knockout mice do not develop colon cancer (66). These results suggest that modifier genes exist that can significantly affect the function of TGFβ1 in preimplantation development, yolk sac development, bowel and gastric inflammation, and colon tumor suppression. Progress toward localizing a modifier gene for the yolk sac developmental problem has been made (67).

What is the best genetic background for knockout mice? Because background-dependent phenotypic variability will likely be found for most knockout mice, it will be useful to backcross a targeted allele onto several mouse backgrounds to make congenic strains. In this section, it will be argued that putting a targeted allele on a mixed strain background will also provide useful information. This is not to say that congenic strains are not useful. Rather, the point to be made here is that there also are benefits to looking at mixed strain backgrounds. Again, our experience with *Tgfb* knockout mice will be instructive.

Generating homozygous mutant knockout animals on a mixed genetic background is faster. The ES cells are nearly always from a 129 strain, and the blastocysts into which the targeted ES cells are injected are nearly always C57BL/6. For reasons unknown, this is a good combination for establishing germline transmission of the injected ES cells. The resulting chimeras can then be crossed with any strain desired, but 129, C57BL/6, or Black Swiss mice are most often used, and CF1 mice were used in the case of our *Tgfb1* knockout mice. Heterozygous offspring from this crossing will then be inbred 129 or F1 hybrids of 129 and one of the other strains. Clearly then, the quickest route to having the knockout allele on an inbred strain is through 129. For the other strains several generations of backcrossing is required, which can take well over a year. Unfortunately, strain-129 mice have low fertility and fecundity. Consequently, the number of offspring per litter is usually fewer than six. Although 129 x C57BL/6 hybrids are more robust, upon backcrossing onto C57BL/6, litter size decreases. To the contrary, the Black Swiss and CF1 strains are robust, and litter size often is in excess of 12. The reason for this is probably because they are not truly inbred strains, but rather are partially outbred through random breeding within their respective strains. Therefore, one of the choices one has is to stay with "pure" genetics at the expense of a lower production rate and considerable delay before generation of experimental animals, or sacrifice some genetic purity to obtain a more efficient production colony. Ideally, one would want to do both, but this often is too expensive.

Mixed genetic background knockout mice often have a wider range of phenotypes. The *Tgfb1* knockout mice backcrossed onto either the 129 or C57BL/6 background (congenics) yield only embryo lethality (63, unpublished observations). On the other hand, when the knockout allele is maintained on mixed genetic backgrounds, embryo and adult phenotypes are maintained.

The *Tgfb2* & *Tgfb3* knockout mice provide further examples. The *Tgfb2* knockout mice have more than two dozen congenital defects and die either immediately preceding or during birth, or within 2 h thereafter (30). Table 2 indicates that most of the phenotypes are only partially penetrant. Though it is not documented, it is likely that the penetrance of some of these phenotypes would increase to nearly 100%, and some of the other phenotypes would disappear were we to put the *Tgfb2* knockout allele on inbred backgrounds. Hence, the mixed strain background probably provided more information than would congenic strains.

The *Tgfb3* knockout mice have a cleft palate (31). One colony of *Tgfb3* knockout mice was left as a mixed background (129 x CF1; 50:50) strain, whereas another colony was backcrossed several generations to the C57BL/6 strain. These two colonies had considerable expressivity differences; the inbred colony had more severe clefting than did the mixed background colony. In the latter, expressivity of clefting varied widely from animal to animal. This variable expressivity within the mixed background colony provided us with the opportunity to obtain far more data on development of the cleft palate and was, therefore, more useful for making assumptions about the cellular and molecular mechanisms by which TGFβ3 supports palate fusion. Hence, using the *Tgfb3* knockout mice, the mixed strain background provided more information than did the congenic strain. Consequently, a wider range of penetrance and expressivity of phenotype is a major advantage of investigating knockout phenotypes in mixed background colonies. Further, variable penetrance of phenotype in a mixed background colony suggests that there are modifier genes for each phenotype that could be obtained by linkage studies.

Conclusions

Questions have been addressed that arose from the last 8 years in which knockout mice have been investigated to analyze gene function at the whole animal level. These questions concern gene redundancy, apparent lack of phenotype in a surprising number of knockout strains, and effects of genetic background on knockout phenotype. Using data obtained principally from *Tgfb* and *Fgf* knockout mice, it is argued that there is probably little redundancy in the genome (i.e., that few genes are dispensable for survival of the species). Apparent lack of phenotype more likely reflects our inability to ask the right questions, or our lack of tools to answer them, than it does a true lack of function. Finally, discussion of genetic background phenotype variability, including variable penetrance and expressivity, was used to present some of the advantages of working with mixed genetic background colonies of knockout mice. For all the examples given here, there are counter examples that must be taken seriously; consequently, these arguments must not be taken as absolutes. For example, if a gene in a particular mouse strain has recently been duplicated, it will most likely be redundant. If one is studying tissue rejection in a knockout mouse, the genetic background obviously must be well defined and preferably inbred. Or, if one wants to use the susceptibility of a particular mouse strain to cancer to investigate the function of the knockout gene in progression of that cancer, the knockout allele must be put on that mouse strain. In general, however, when setting up approaches for investigating a new gene knockout mouse, I believe one would be well advised to assume that: there is little gene redundancy in mammals; there are knockout phenotypes even if none are immediately apparent; and investigating phenotypes in mixed genetic background colonies may not only reveal more phenotypes, but may lead to better understanding of the molecular or cellular mechanism underlying the phenotype, and may lead to modifier gene discovery.

Acknowledgements

I thank all the members of the Doetschman lab for their intellectual stimulation, for the constant generation of exciting experimental results, and for their ability to become intrigued rather than discouraged when those results are not what was expected.

The work discussed here was supported by grant nos. HD26471, HL58511, HL41496, ES05652, ES06096, and AR44059 to T. D.

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MOLECULAR BIOLOGY OF THE CELL

fourth edition

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Library of Congress Cataloging-in-Publicaton Data
Molecular biology of the cell / Bruce Alberts ... [et al.].-- 4th ed.
p. cm
Includes bibliographical references and index.
ISBN 0-8153-3218-1 (hardbound) -- ISBN 0-8153-4072-9 (pbk.)
1. Cytology. 2. Molecular biology. I. Alberts, Bruce.
[DNLM: 1. Cells. 2. Molecular Biology.]
QH581.2 .M64 2002
571.6--dc21

2001054471 CIP

Published by Garland Science, a member of the Taylor & Francis Group, 29 West 35th Street, New York, NY 10001-2299

Printed in the United States of America

15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

Front cover Human Genome: Reprinted by permission from *Nature*, International Human Genome Sequencing Consortium, 409:860–921, 2001 © Macmillan Magazines Ltd. Adapted from an image by Francis Collins, NHGRI; Jim Kent, UCSC; Ewan Birney, EBI; and Darryl Leja, NHGRI; showing a portion of Chromosome 1 from the initial sequencing of the human genome.

Back cover In 1967, the British artist Peter Blake created a design classic. Nearly 35 years later Nigel Orme (illustrator), Richard Denyer (photographer), and the authors have together produced an affectionate tribute to Mr Blake's image. With its gallery of icons and influences, its assembly created almost as much complexity, intrigue and mystery as the original. *Drosophila*, *Arabidopsis*, Dolly and the assembled company tempt you to dip inside where, as in the original, "a splendid time is guaranteed for all." (Gunter Blobel, courtesy of The Rockefeller University; Marie Curie, Keystone Press Agency Inc; Darwin bust, by permission of the President and Council of the Royal Society; Rosalind Franklin, courtesy of Cold Spring Harbor Laboratory Archives; Dorothy Hodgkin, © The Nobel Foundation, 1964; James Joyce, etching by Peter Blake; Robert Johnson, photo booth self-portrait early 1930s, © 1986 Delta Haze Corporation all rights reserved, used by permission; Albert L. Lehninger, (unidentified photographer) courtesy of The Alan Mason Chesney Medical Archives of The Johns Hopkins Medical Institutions; Linus Pauling, from Ava Helen and Linus Pauling Papers, Special Collections, Oregon State University; Nicholas Poussin, courtesy of ArtToday.com; Barbara McClintock, © David Micklos, 1983; Andrei Sakharov, courtesy of Elena Bonner; Frederick Sanger, © The Nobel Foundation, 1958.)

the target gene in a particular place or at a particular time. The most common of these recombination systems called **Cre/lox**, is widely used to engineer gene replacements in mice and in plants (see Figure 5–82). In this case the target gene in ES cells is replaced by a fully functional version of the gene that is flanked by a pair of the short DNA sequences, called lox sites, that are recognized by the Cre recombinase protein. The transgenic mice that result are phenotypically normal. They are then mated with transgenic mice that express the Cre recombinase gene under the control of an inducible promoter. In the specific cells or tissues in which Cre is switched on, it catalyzes recombination between the lox sequences—excising a target gene and eliminating its activity. Similar recombination systems are used to generate conditional mutants in *Drosophila* (see Figure 21–48).

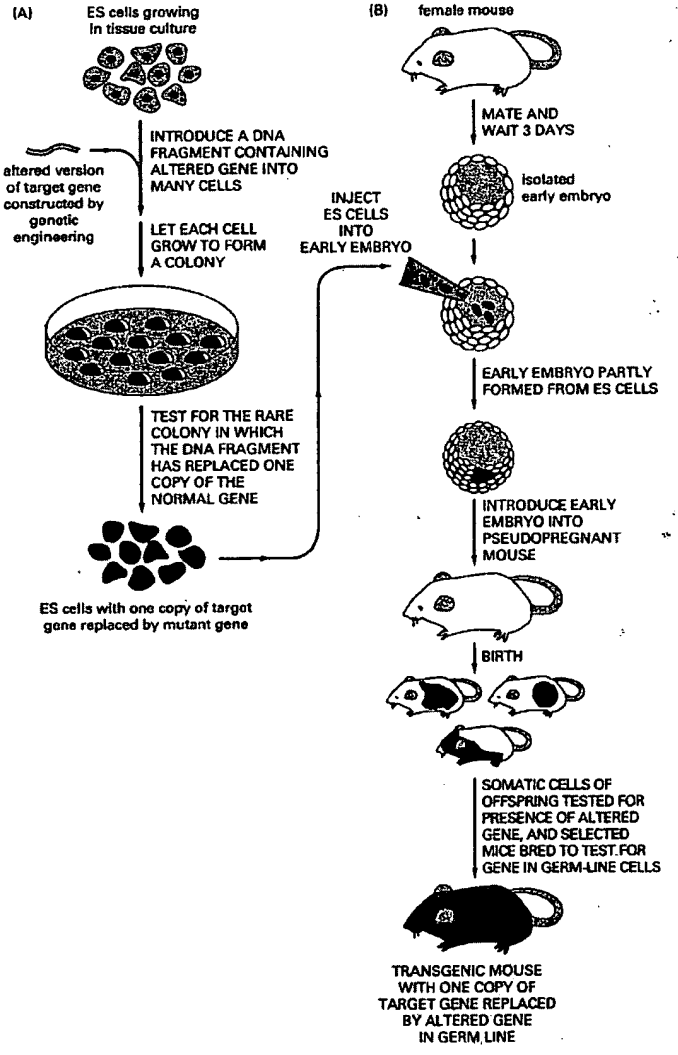

Figure 8–70 Summary of the procedures used for making gene replacements in mice. In the first step (A), an altered version of the gene is introduced into cultured ES (embryonic stem) cells. Only a few rare ES cells will have their corresponding normal genes replaced by the altered gene through a homologous recombination event. Although the procedure is often laborious, these rare cells can be identified and cultured to produce many descendants, each of which carries an altered gene in place of one of its two normal corresponding genes. In the next step of the procedure (B), these altered ES cells are injected into a very early mouse embryo; the cells are incorporated into the growing embryo, and a mouse produced by such an embryo will contain some somatic cells (indicated by *orange*) that carry the altered gene. Some of these mice will also contain germ-line cells that contain the altered gene. When bred with a normal mouse, some of the progeny of these mice will contain the altered gene in all of their cells. If two such mice are in turn bred (not shown), some of the progeny will contain two altered genes (one on each chromosome) in all of their cells.

If the original gene alteration completely inactivates the function of the gene, these mice are known as knockout mice. When such mice are missing genes that function during development, they often die with specific defects long before they reach adulthood. These defects are carefully analyzed to help decipher the normal function of the missing gene.

Transgenic Plants Are Important for Both Cell Biology and Agriculture

When a plant is damaged, it can often repair itself by a process in which mature differentiated cells "dedifferentiate," proliferate, and then redifferentiate into other cell types. In some circumstances the dedifferentiated cells can even form an apical meristem, which can then give rise to an entire new plant, including gametes. This remarkable plasticity of plant cells can be exploited to generate transgenic plants from cells growing in culture.

When a piece of plant tissue is cultured in a sterile medium containing nutrients and appropriate growth regulators, many of the cells are stimulated to proliferate indefinitely in a disorganized manner, producing a mass of relatively undifferentiated cells called a callus. If the nutrients and growth regulators are carefully manipulated, one can induce the formation of a shoot and then root apical meristems within the callus, and, in many species, a whole new plant can be regenerated.

Callus cultures can also be mechanically dissociated into single cells, which will grow and divide as a suspension culture. In several plants—including tobacco, petunia, carrot, potato, and *Arabidopsis*—a single cell from such a suspension culture can be grown into a small clump (a clone) from which a whole plant can be regenerated. Such a cell, which has the ability to give rise to all parts of the organism, is considered **totipotent.** Just as mutant mice can be derived by genetic manipulation of embryonic stem cells in culture, so transgenic plants can be created from single totipotent plant cells transfected with DNA in culture (Figure 8–72).

The ability to produce transgenic plants has greatly accelerated progress in many areas of plant cell biology. It has had an important role, for example, in isolating receptors for growth regulators and in analyzing the mechanisms of morphogenesis and of gene expression in plants. It has also opened up many new possibilities in agriculture that could benefit both the farmer and the consumer. It has made it possible, for example, to modify the lipid, starch, and protein storage reserved in seeds, to impart pest and virus resistance to plants, and to create modified plants that tolerate extreme habitats such as salt marshes or water-stressed soil.

Many of the major advances in understanding animal development have come from studies on the fruit fly *Drosophila* and the nematode worm *Caenorhabditis elegans*, which are amenable to extensive genetic analysis as well as to experimental manipulation. Progress in plant developmental biology has, in the past, been relatively slow by comparison. Many of the plants that have proved most amenable to genetic analysis—such as maize and tomato—have long life cycles and very large genomes, making both classical and molecular genetic analysis time-consuming. Increasing attention is consequently being paid to a fast-growing small weed, the common wall cress *(Arabidopsis thaliana)*, which has several major advantages as a "model plant" (see Figures 1–46 and 21–107). The relatively small *Arabidopsis* genome was the first plant genome to be completely sequenced.

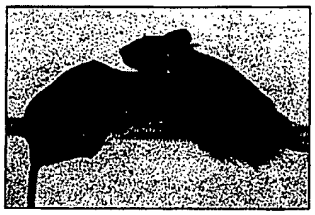

Figure 8–71 Mouse with an engineered defect in fibroblast growth factor 5 (FGF5). FGF5 is a negative regulator of hair formation. In a mouse lacking FGF5 *(right)*, the hair is long compared with its heterozygous littermate *(left)*. Transgenic mice with phenotypes that mimic aspects of a variety of human disorders, including Alzheimer's disease, atherosclerosis, diabetes, cystic fibrosis, and some type of cancers, have been generated. Their study may lead to the development of more effective treatments. (Courtesy of Gail Martin, from J.M. Hebert et al., *Cell* 78:1017–1025, 1994. © Elsevier.)

Large Collections of Tagged Knockouts Provide a Tool for Examining the Function of Every Gene in an Organism

Extensive collaborative efforts are underway to generate comprehensive libraries of mutations in several model organisms, including *S. cerevisiae, C. elegans, Drosophila, Arabidopsis*, and the mouse. The ultimate aim in each case is to produce a collection of mutant strains in which every gene in the organism has either been systematically deleted, or altered such that it can be conditionally disrupted. Collections of this type will provide an invaluable tool for investigating gene function on a genomic scale. In some cases, each of the individual mutants within the collection will sport a distinct molecular tag—a unique DNA sequence designed to make identification of the altered gene rapid and routine.

In *S. cerevisiae*, the task of generating a set of 6000 mutants, each missing

Genes VII

Benjamin Lewin

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Published in the United States
by Oxford University Press Inc., New York

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First published 2000

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A catalogue record for this book is available from the British Library

Library of Congress Cataloging in Publication Data
(Data applied for)

ISBN 0-19-879276-X (Hbk)

Typeset by J&L Composition Ltd, Filey, North Yorkshire
Printed in The United States of America

which exogenous DNA is introduced from a bacterium into a host cell. The mechanism resembles that of bacterial conjugation. Expression of the bacterial DNA in its new host changes the phenotype of the cell. In the example of the bacterium *Agrobacterium tumefaciens*, the result is to induce tumor formation by an infected plant cell.

Alterations in the relative proportions of components of the genome during somatic development occur to allow insect larvae to increase the number of copies of certain genes. And the occasional amplification of genes in cultured mammalian cells is indicated by our ability to select variant cells with an increased copy number of some genes. Initiated within the genome, the amplification event can create additional copies of a gene that survive in either intrachromosomal or extrachromosomal form.

When extraneous DNA is introduced into eukaryotic cells, it may give rise to extrachromosomal forms or may be integrated into the genome. The relationship between the extrachromosomal and genomic forms is irregular, depending on chance and to some degree unpredictable events, rather than resembling the regular interchange between free and integrated forms of bacterial plasmids.

Yet, however accomplished, the process may lead to stable change in the genome; following its injection into animal eggs, DNA may even be incorporated into the genome and inherited thereafter as a normal component, sometimes continuing to function. Injected DNA may enter the germline as well as the soma, creating a transgenic animal. The ability to introduce specific genes that function in an appropriate manner could become a major medical technique for curing genetic diseases.

The converse of the introduction of new genes is the ability to disrupt specific endogenous genes. Additional DNA can be introduced within a gene to prevent its expression and to generate a null allele. Breeding from an animal with a null allele can generate a homozygous "knockout", which has no active copy of the gene. This is a powerful method to investigate directly the importance and function of a gene.

Considerable manipulation of DNA sequences therefore is achieved both in authentic situations and by experimental fiat. We are only just beginning to work out the mechanisms that permit the cell to respond to selective pressure by changing its bank of sequences or that allow it to accommodate the intrusion of additional sequences.

The mating pathway is triggered by signal transduction

THE yeast *S. cerevisiae* can propagate happily in either the haploid or diploid condition. Conversion between these states takes place by mating (fusion of haploid spores to give a diploid) and by sporulation (meiosis of diploids to give haploid spores). The ability to engage in these activities is determined by the mating type of the strain.

The properties of the two mating types are summarized in Figure 17.1. We may view them as resting on the teleological proposition that there is no point in mating unless the haploids are of different genetic types; and sporulation is productive only when the diploid is heterozygous and thus can generate recombinants.

The mating type of a (haploid) cell is determined by the genetic information present at the *MAT* locus. Cells that carry the *MATa* allele at this locus are type a; likewise, cells that carry the *MATα* allele are type α. Cells of opposite type can mate; cells of the same type cannot.

Recognition of cells of opposite mating type is

Figure 17.1 Mating type controls several activities.

	MATa	*MATα*	*MATa/MATα*
Cell type	a	α	a/α
Mating	yes	yes	no
Sporulation	no	no	yes
Pheromone	a factor	α factor	none
Receptor	binds α factor	binds a factor	none

Gene Targeting

A Practical Approach

Second Edition

Edited by

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OXFORD
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Great Clarendon Street, Oxford OX2 6DP
Oxford University Press is a department of the University of Oxford and furthers the University's aim of excellence in research, scholarship, and education by publishing worldwide in
Oxford New York
Auckland Bangkok Buenos Aires Cape Town
Chennai Dar es Salaam Delhi Hong Kong
Istanbul Karachi Kolkata Kuala Lumpur Madrid
Melbourne Mexico City Mumbai Nairobi
São Paulo Shanghai Taipei Tokyo Toronto
Oxford is a registered trade mark of Oxford University Press
Published in the United States
by Oxford University Press Inc., New York
© Oxford University Press, 2000
First published 2000
Reprinted 2001, 2003
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A catalogue record for this book is available from the British Library

Library of Congress Cataloging in Publication Data

Gene targeting : a practical approach / edited by Alexandra L. Joyner.
—2nd ed.
Includes bibliographical references and index.
1. Gene targeting Laboratory manuals. I. Joyner, Alexandra L.
QH442.3.G456 1999 660.6'5—dc21 99–36660

ISBN 0-19-963792-X (Pbk)

Printed in Great Britain by Information Press, Ltd,
Eynsham, Oxon.

Preface

Over the past ten years it has become possible to make essentially any mutation in the germline of mice by utilizing recombination and embryonic stem (ES) cells. Homologous recombination when applied to altering specific endogenous genes, referred to as gene targeting, provides the highest level of control over producing mutations in cloned genes. When this is combined with site specific recombination, a wide range of mutations can be produced. ES cell lines are remarkable since after being established from a blastocyst, they can be cultured and manipulated relatively easily in vitro and still maintain their ability to step back into a normal developmental program when returned to a pre-implantation embryo. With the exponential increase in the number of genes identified by various genome projects and genetic screens, it has become imperative that efficient methods be developed for determining gene function. Gene targeting in ES cells offers a powerful approach to study gene function in a mammalian organism. Gene trap approaches in ES cells, in particular when they are combined with sophisticated prescreens, offer not only a route to gene discovery, but also to gain information on gene sequence, expression and mutant phenotype.

The basic technology necessary for making designer mutations in mice has become widespread and researchers who have traditionally used cell biology or molecular experiments are adding gene targeting techniques to their repertoire of experimtal approsches. A second edition of this book was written for two main reasons. The first was to update previously described techniques and to add new techniques that have greatly expanded the types of mutations that can be made using recombination in ES cells. A chapter in this new edition describes the design and use of site specific recombination for gene targeting approaches and production of conditional mutations. The second reason for the new book was to provide a more in depth discussion of the experimental design considerations that are critical to a successful gene targeting study and to add approaches for analyzing mutant phenotypes, the most interesting part of an experiment. Gene targeting experiments should be designed to go far beyond just making a mutant mouse. The success of a gene targeting experiment no longer lies in the making of the mutation, but depends on the imaginative and insightful analysis of the mutant phenotypes that the mutation provides. A chapter in this edition describes the use of classical genetics in combination with gene targeting to get the most out of a genetic approach to a biological question.

The nature of in vivo gene targeting studies of gene function are such that critical design decisions must be made at every step in the experiment, and each decision can have a major impact on the value of the information obtained. From the start, the type of mutation to be made must be considered

carefully. Whereas 10 years ago most mutations were designed to create null mutations and were therefore relatively simple to design, at present, a null mutation is only one of a long list of mutations that can be made, each providing different insight into the function of a gene. Point mutations, large deletions, gene exchanges (knock-ins) and conditional mutations are but a few of the choices one faces at the start of a gene targeting experiment. The next choice is the source of DNA for the targeting experiment and ES cell line to be used for the manipulations. Once the mutant ES cell clone has been obtained, there are then a number of alternative approaches that can be used to make ES cell chimeras that depend on the ES cell line which was used. Finally, and most importantly, is the analysis of any phenotype that arises. This second addition discusses techniques used to analyze mutant mice, ranging from standard descriptive evaluation, to a chimera analysis or complicated breeding experiments that utilize double mutants. If mice are simply considered as a 'bag of cells' or an in vivo source of selected cell types, then the tremendous resource which mice offer as a model organism is not being realized. The life of a mouse represents a continuum of dynamic processes, including pattern formation, organ development, learning, homeostasis and disease. By making genetic alterations in mice using gene targeting and ES cells, the effects of a given change can be studied in the context of the whole organism.

My goal in editing this book was to provide a manual that could take a newcomer to the exciting field of gene targeting and mutant analysis in mice from a cloned gene to a basic understanding of the genetic approaches available using ES cells, and how each technique can be used to design a particular in vivo test of gene function. The book should also provide a valuable bench side resource for anyone carrying out gene targeting or gene trap experiments, a chimera analysis or classical genetic approaches. I would once again like to extend many thanks and my deepest appreciation to all the authors for their great efforts in including detailed protocols and lucid discussions of the various approaches presented. I would also like to thank my family for their strong support and laboratory members past and present for helping to make gene targeting a reality. Finally, since many of the techniques use mice, the experiments should be carried out in accordance with local regulations.

New York, NY A.L.J.

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Gene targeting, principles, and practice in mammalian cells

PAUL HASTY, ALEJANDRO ABUIN, and ALLAN BRADLEY

1. Introduction

When a fragment of genomic DNA is introduced into a mammalian cell it can locate and recombine with the endogenous homologous sequences. This type of homologous recombination, known as gene targeting, is the subject of this chapter. Gene targeting has been widely used, particularly in mouse embryonic stem (ES) cells, to make a variety of mutations in many different loci so that the phenotypic consequences of specific genetic modifications can be assessed in the organism.

The first experimental evidence for the occurrence of gene targeting in mammalian cells was made using a fibroblast cell line with a selectable artificial locus by Lin *et al.* (1), and was subsequently demonstrated to occur at the endogenous β-globin gene by Smithies *et al.* in erythroleukaemia cells (2). In general, the frequencies of gene targeting in mammalian cells are relatively low compared to yeast cells and this is probably related to, at least in part, a competing pathway: efficient integration of the transfected DNA into a random chromosomal site. The relative ratio of targeted to random integration events will determine the ease with which targeted clones are identified in a gene targeting experiment. This chapter details aspects of vector design which can determine the efficiency of recombination, the type of mutation that may be generated in the target locus, as well as the selection and screening strategies which can be used to identify clones of ES cells with the desired targeted modification. Since the most common experimental strategy is to ablate the function of a target gene (*null allele*) by introducing a selectable marker gene, we initially describe the vectors and the selection schemes which are helpful in the identification of recombinant clones (Sections 2–5). In Section 6, we describe the vectors and additional considerations for generating subtle mutations in a target locus devoid of any exogenous sequences. Finally, Section 7 is dedicated to the use of gene targeting as a method to express exogenous genes from specific endogenous regulatory elements *in vivo*, also known as 'knock-in' strategies.

enrich populations of transfected cells for targeted integration events (Section 4.2.1).

2.1 Design considerations of a replacement vector

The principal consideration in the design of a replacement vector, is the type of mutation generated. Secondary (yet still important) considerations relate to the selection scheme and screening techniques required to isolate the recombinant clones. The recombinant alleles generated by replacement vectors typically have a selection cassette inserted into a coding exon or replacing part of the locus. It is important to consider that, exon interruptions and small deletions will not necessarily ablate the function of the target gene to generate a null allele. Consequently, it is necessary to confirm that the allele which has been generated is null by RNA and/or protein analysis and in many cases transcripts and truncated proteins from such a mutant allele can be detected. Considering that products from the mutated locus may have some function (normal or abnormal) it is important to design a replacement vector so that the targeted allele is null, particularly in the absence of a good assay for the gene product. Disruption or deletion of the coding sequence by the positive selection marker will in most instances ablate a gene's function. However in some situations a truncated protein may be generated which retains some biological activity, thus some knowledge of mutations in a related gene in another organism can be helpful in the determination of the possible function of a targeted allele. Null alleles are more likely to occur by deleting or recombining a selection cassette into more 5' exons rather than exons that encode the C-terminus of the protein, since under these circumstances minimal portions of the wild-type polypeptide would be made.

There are several considerations to take into account when a positive selection marker is to be inserted into an exon. One critical consideration is that since the length of an exon can influence RNA splicing (3), an artificially large exon caused by the insertion of a selectable marker may not be recognized by the splicing machinery and could be skipped. Thus, transcripts initiated from the endogenous promoter may delete the mutated exon from the mRNA species or even additional exons. If a skipped exon is a coding exon whose nucleotide length is not a multiple of three (codon) the net result will be both a deletion and a frame-shift mutation of the gene, which will often generate a null allele. However, if the disrupted coding exon has a nucleotide length which is a multiple of three, if spliced out, this would result in a protein with a small in-frame deletion which may retain partial or complete function. The same concept applies to gene targeting vectors in which exons are being deleted and replaced by the selectable marker. Deletion of an exon or group of exons with a unit number of codons may also result in a functional protein product with an in-frame deletion. For most purposes it is advisable to delete portions or all of the target gene so that the genetic

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Production of targeted embryonic stem cell clones

MICHAEL P. MATISE, WOJTEK AUERBACH and ALEXANDRA L. JOYNER

1. Introduction

The discovery that cloned DNA introduced into tissue culture cells can undergo homologous recombination at specific chromosomal loci has revolutionized our ability to study gene function in cell culture and *in vivo*. In theory, this technique, termed gene targeting, allows one to generate any type of mutation in any cloned gene. The kinds of mutations that can be created include null mutations, point mutations, deletions of specific functional domains, exchanges of functional domains from related genes, and gain-of-function mutations in which exogenous cDNA sequences are inserted adjacent to endogenous regulatory sequences. In principle, such specific genetic alterations can be made in any cell line growing in culture. However, not all cell types can be maintained in culture under the conditions necessary for transfection and selection. Over ten years ago, pluripotent embryonic stem (ES) cells derived from the inner cell mass (ICM) of mouse blastocyst stage embryos were isolated and conditions defined for their propogation and maintenance in culture (1, 2). ES cells resemble ICM cells in many respects, including their ability to contribute to all embryonic tissues in chimeric mice. Using stringent culture conditions, the embryonic developmental potential of ES cells can be maintained following genetic manipulations and after many passages *in vitro*. Furthermore, permanent mouse lines carrying genetic alterations introduced into ES cells can be obtained by transmitting the mutation through the germline by generating ES cell chimeras (described in Chapters 4 and 5). Thus, applying gene targeting technology to ES cells in culture affords researchers the opportunity to modify endogenous genes and study their function *in vivo*. In initial studies, one of the main challenges of gene targeting was to distinguish the rare homologous recombination events from more commonly occurring random integrations (discussed in Chapter 1). However, advances in cell culture and in selection schemes, in vector construction using isogenic DNA, and in the application of rapid screening procedures have made it possible to identify homologous recombination events efficiently.

What's Wrong With My Mouse?

Behavioral Phenotyping of Transgenic and Knockout Mice

Jacqueline N. Crawley, Ph.D.

WILEY-LISS

A John Wiley & Sons, Inc., Publication

New York • Chichester • Weinheim • Brisbane • Singapore • Toronto

This book was written by Dr. Jacqueline Crawley in her private capacity, outside of her professional position as Chief, Section on Behavioral Neuropharmacology, National Institute of Mental Health, Bethesda, Maryland, USA. The views expressed in this book do not necessarily represent the views of the National Institutes of Health or of the United States government.

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Published simultaneously in Canada.

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Library of Congress Cataloging-in-Publication Data:
Crawley, Jacqueline N.
What's wrong with my mouse? : behavioral phenotyping of transgenic and knockout mice / Jacqueline N. Crawley.
p. cm.
Includes bibliographical references.
ISBN 0-471-31639-3 (cloth : alk. paper)
1. Neurogenetics—Animal models. 2. Transgenic mice—Behavior.
I. Title.
QP356.22.C73 1999
573.8'48'0724—dc21 99-28955

Printed in the United States of America.
10 9 8 7 6 5 4

Preface

Targeted mutation of genes expressed in the nervous system is an exciting new research field that is forging a remarkable amalgam of molecular genetics and behavioral neuroscience. My laboratory in Bethesda has been the fortunate recipient of visits from many molecular geneticists over the past five years, who come to ask, "What's wrong with my mouse? Can you tell us what behaviors are abnormal in our null mutants? And how *do* you measure behavior, anyway?"

We have had some remarkable opportunities to collaborate with outstanding molecular geneticists in the National Institutes of Health Intramural Research Program and throughout the world on investigations of the behavioral effects of mutations in genes expressed in the mouse brain. Each of these collaborations has been a learning experience, increasing our understanding of the optimal experimental design for analyzing behavioral phenotypes of mutant mice. What are the best tests to address each specific hypothesis? Which methods work best for mice? Which rat tasks can be adapted for mice? What are the correct controls? What are the hidden pitfalls, lurking artifacts, false positives, and false negatives? Which statistical tests are most sensitive for detection of the genotype effect? What is the minimum number of animals necessary for each genotype, gender, and age? Our laboratory and many others are gradually working out the best methods for behavioral phenotyping of transgenic and knockout mice.

In the same conversations, molecular geneticists frequently asked me to recommend a book they could consult to learn more about behavioral tests for mice. Apparently the scientific book publishers are receiving similar queries. Ann Boyle and Robert Harington at John Wiley & Sons, convinced of a real need for such a book, sweet-talked me into filling the void. *What's Wrong With My Mouse?* is written for these pioneering molecular geneticists, and for the talented students who will be the next driving force in moving the field forward.

On a personal level, I would like to express deep appreciation to all of my behavioral neuroscientist colleagues around the world for their outstanding work, past, present, and future. Your contributions to the excellence and abundance of mouse behavioral tests provide

the foundation for the rapidly expanding scientific discoveries forthcoming from behavioral phenotyping studies of transgenic and knockout mice. This book is a testament to your accomplishments.

JACQUELINE N. CRAWLEY, PH.D.
Chevy Chase, Maryland

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Designer Mice

The disease is inherited. Family pedigrees indicate an autosomal dominant gene. Linkage analyses reveal one strongly associated chromosomal locus. Mapping identifies the gene. The cDNA for the gene is sequenced. The anatomical distribution of the gene is primarily in the brain. The symptoms of the disease are primarily neuropsychiatric. There is no treatment for the disease. The disease is lethal.

Your mission, should you choose to accept it, is to develop a treatment for the disease. Replacement gene therapy is the best hope. But you don't know the gene product, you don't know its function, and you don't know if gene delivery would be therapeutic. Where do you start?

These days, you may choose to start with a targeted gene mutation, to generate a mutant mouse model of the hereditary disease. A DNA construct containing the mutated form of the responsible gene is developed. The construct is inserted into the mouse genome. A line of mice with the mutated gene is generated. Characteristics of the mutant mice are identified in comparison to normal controls. Salient characteristics relevant to the human disease are quantitated. These diseaselike traits are then used as test variables for evaluating the effectiveness of treatments. Putative treatments are administered to the mutant mice. A treatment that prevents or reverses the disease traits in the mutant mice is taken forward for further testing as a potential therapeutic treatment for the human genetic disease. Gene therapy, based on targeted gene replacement of the missing or incorrect gene in the human hereditary disease, is described in Chapter 12. In the future, medicine may shift emphasis from treating the symptoms to administering replacement genes that effectively and permanently cure the disease.

Targeted gene mutation in mice represents a new technology that is revolutionizing biomedical research. **Transgenic** mice have an ***extra gene*** added. An additional copy of a normal gene is inserted into the mouse genome to study overexpression of the gene product. Or a new gene is added that is not normally present in the mouse genome. The new gene may be the aberrant form of a human gene linked to a disease. For example, the mutated form of the human *huntingtin* gene is added to the mouse genome to generate a mouse model of Huntington's disease. **Knockout** mice have a ***gene deleted***. The **null mutant homozygous**

knockout mouse is deficient in both alleles of a gene; the heterozygote is deficient in one of its two alleles for the gene. The genotype is −/− for the null mutant, +/− for the heterozygote, and +/+ for the wildtype normal control. The phenotype is the set of observed characteristics resulting from the mutation. Phenotypes include biochemical, anatomical, physiological, and behavioral characteristics.

Targeted mutations of genes expressed in the brain are revealing the mechanisms underlying normal behavior and behavioral abnormalities. Mouse models of human neuropsychiatric diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, obesity, anorexia, depression, alcoholism, drug addiction, schizophrenia, and anxiety, are likely to be characterized by their behavioral phenotype.

This book is designed to introduce the novice to the rich literature of behavioral tests in mice and to show how to optimize the application of these tests for behavioral phenotyping of mutant mice. Based on our experiences, our laboratory is working toward a unified approach for the optimal conduct of behavioral phenotyping experiments in mutant mice. Recommendations are offered for a three-tiered sequence of behavioral tests, applicable to each behavioral domain relevant to genes expressed in the mammalian brain.

SCOPE

This book is designed as an overview of the mutant mouse technology and an introducion to the field of behavioral neuroscience, as it can be applied to behavioral phenotyping of transgenic and knockout mice. Molecular geneticists may browse through the chapters relevant to their gene, to get ideas for possible tests to try. Behavioral neuroscientists who have no experience with mutant mice may wish to read about the methods for developing a transgenic or knockout, the behavioral tests that have been effectively applied, and some of the successful experiments published in the genetics literature.

Chapters are organized around behavioral domains, including general health, neurological reflexes, developmental milestones, motor functions, sensory abilities, learning and memory, feeding, sexual and parental behaviors, social behaviors, and rodent paradigms relevant to fear, anxiety, depression, schizophrenia, reward, and drug addiction. Each chapter begins with a brief history of the early work in the field and the present hypotheses about mechanisms underlying the expression of the behavior. A list of general review articles and books is offered for each topic, encouraging the interested reader to gain more in-depth knowledge of the relevant literature.

Standard tests are then presented in detail. Highlighted are those tasks that have been extensively validated in mice. Demonstrations of genetic components of task performance are described, including experiments comparing inbred strains of mice (strain distributions), quantitative trait loci approaches (linkage analysis), and naturally occurring mutants (spontaneous mutations). Experimental design and specific behavioral tasks are presented as simply as possible. Extensive references are included for each behavioral test to obtain more complete methods from the primary experimental literature on the topic.

Illustrations are provided for the most frequently used behavioral tasks. Photographs of the equipment or diagrams of the task accompany the text. Samples of data are shown. The data presentation is designed to indicate the qualitative and quantitative results that can be expected when the task is properly conducted.

Each chapter includes the results of several representative experiments in which these tasks are successfully applied to characterize transgenic and knockout mice. Examples are

WHAT'S WRONG WITH MY MOUSE?

Transgenic and knockout mutations provide an important means for understanding gene function, as well as for developing therapies for genetic diseases. This engaging and informative book discusses the many advances in the field of transgenic technology that have enabled researchers to bring about various changes in the mouse genome. Equal emphasis is given to both the principles of transgenic and knockout methods and their applications. A clear and concise format provides researchers with a comprehensive review of the behavioral paradigms appropriate for analyzing mouse phenotypes.

What's Wrong with My Mouse? explains the differences between transgenic knockout mice and their wild-type controls, while providing critical information about gene function and expression. This volume recognizes that newly identified genes can provide useful insights into brain functioning, including brain malfunctioning in disease states. Written by a world-renowned expert in the field, the material also covers:

- How to generate a transgenic or knockout mouse
- Motor functions (open field, holeboard, rotarod, balance, grip, circadian activity, etc.)
- Sensory abilities (olfaction, vision, hearing, taste, touch, nociception)
- Reproductive behavior, social behavior, and emotional behavior

Researchers in neuroscience, pharmacology, genetics, developmental biology, and cell biology will all find this book essential reading.

Cover Design: Paul DiNovo

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Industrializing breakthrough discovery

Arthur T Sands
Lexicon Genetics, USA

Predicting drug action using mouse knockouts was pioneered by Lexicon; five years later the full potential of gene knockout technology is beginning to be realized. In combination with comprehensive physiological analysis, the technology delivers novel, *in vivo*-validated targets with the potential for the discovery of breakthrough therapeutics.

Innovation in the pharmaceutical industry depends on breakthrough biological discoveries that reveal new targets for therapeutic intervention. These new targets must provide potent new mechanisms of action to block disease by creating favorable alterations in physiology without undesirable side effects. *In vivo* methods of target validation using gene knockouts have revealed truly rare and valuable targets. This fact stands in direct contrast to the popular myth that the human genome contains thousands of viable drug targets. It seems that a simple truth has been all but forgotten in the pursuit of ultra-high-throughput drug discovery: it is the quality of new targets not the quantity that holds promise for replenishing the pharmaceutical industry's drug discovery pipeline.

There is plenty of evidence that more is not necessarily better. The pharmaceutical industry spends $30 billion each year on research and development - three times more than a decade ago - yet the number of new drugs coming to market has not increased. The industry's product innovation bottleneck is especially critical since $38.6 billion in brand name drugs will be coming off patent over the course of the next three years, creating a market void that drug makers are not prepared to fill.

Physiology must guide discovery

To replenish product pipelines, the industry is looking to biotechnology companies to accelerate the identification and validation of new targets. In order to discover which genes among thousands encode breakthrough targets, industry scientists must conduct rigorous physiological assessments to determine which targets to eliminate and which to pursue. Only those targets that demonstrate the potential to maximize therapeutic effects and minimize side effects should be pursued, thereby reducing the failure rate and increasing the overall efficiency of the drug discovery process.

Since a therapeutic alteration in physiology is the desired endpoint of drug discovery, overly reductionist approaches that ignore the complexity of mammalian physiology are inevitably doomed to failure. Computer modeling, DNA microarrays, proteomics and lower model organisms cannot encompass the complexity of mammalian physiology and may actually distract researchers from a more productive pathway to discovery. Even human genetic studies may be problematic, since they are more likely to reveal genes that cause disease rather than drug targets for future cures.

Just as drugs must act within the context of physiology, novel drug targets must be validated within the context of mammalian physiology before precious resources are expended to develop drugs. Grounding genomics in the discipline of physiology can increase success rates, enhance product pipelines and create safer and superior therapeutics, as well as reduce the enormous amount of time and capital expended for the discovery and development of a drug. Those companies who are equipped to rapidly and effectively integrate physiological information into the target selection process will dominate the next generation of successful drug discoveries.

Of knockout mice and men

After a decade of using mouse knockouts, the data on their predictive power in drug discovery is irrefutable. The top 100 selling drugs in 2001 are directed only to 29 drug targets, many with multiple agents addressing the same target. Of these 29 targets, 23 have been knocked out and in every case the knockout mouse was highly predictive as to the on-target effects and side effects of the associated drugs. These observations lay to rest early theoretical concerns regarding the reliability of the mouse knockout technology to recapitulate actions of drugs in mammalian model systems. The recent near completion of the genomic sequence of mouse and man, now available through either public or private DNA sequence databases, has confirmed the high rate of genomic similarity between the two organisms. Indeed, many decades of research have proved the mouse to be an invaluable tool for the evaluation of biological processes relevant to human disease, including immunology, oncology, neurobiology, cardiovascular biology, obesity and many others. Well-established parallels exist between humans and mice on cellular, biochemical and physiological levels.

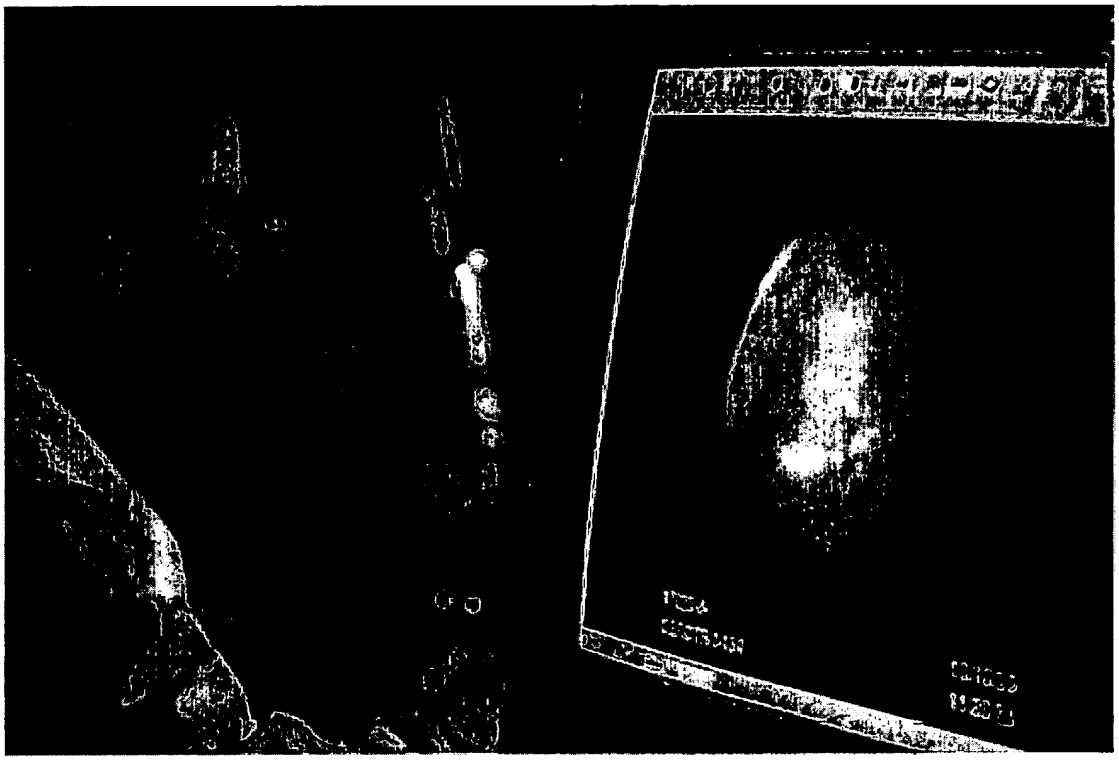
Industrializing discovery

At Lexicon Genetics mouse knockouts are guiding researchers to discover new therapeutic agents which represent the best

physiologic switches in the genome for the treatment of disease. This has required the industrialization of gene targeting, gene trapping and mouse embryonic stem cell technologies, as well as the build-up of significant scientific infrastructure. This infrastructure will allow the company to analyze 5000 genes as mouse knockouts in its Genome5000 program over the next five years. Efforts are concentrated on the unknown function of known gene families for which therapeutic agents can be developed through small molecule chemistry, antibody or therapeutic protein development. These gene families include G protein-coupled receptors, kinases, proteases, ion channels, secreted proteins, transporters and other key enzyme classes. Gene targeting by homologous recombination combined with gene trapping maximizes both selectivity and throughput for large-scale, *in vivo* target validation.

> ***"It seems that a simple truth has been all but forgotten in the pursuit of ultra-high-throughput drug discovery: it is the quality of new targets not the quantity that holds promise for replenishing the pharmaceutical industry's drug discovery pipeline."***

The company has deployed a comprehensive, *in vivo* analysis of candidate drug targets that has been modeled after clinical evaluation. Genes analyzed in this way are subject to a superior level of *in vivo* analysis, including physiological function and potential disease indication, providing a robust pipeline of high-value targets. This approach has already proved successful in extracting vital information about the potential medical utility of several new targets in atherosclerosis, diabetes, obesity and CNS disease, among others. Lexicon's physiological analysis utilizes a wide range of the latest medical technologies, including intensive analytical procedures such as the CAT scan for organ system visualization, dual energy X-ray absorptiometry for measurement of percentage fat and lean body mass and bone mineral density, functional magnetic resonance imaging, which allows *in vivo* neurochemical and cardiac analysis, clinical blood and urine chemistries, complete blood cell counts, fluorescent-activated cell sorting, cell-cycle analysis and neurobehavioral testing. Histopathological and gene expression surveys of 55 tissues provide cellular and gene expression data for additional information. Disease challenge models may also be used when indicated to maintain a



Computer modeling, DNA microarrays, proteomics and lower model organisms cannot encompass the complexity of mammalian physiology and may actually distract researchers from a more productive pathway to discovery.

high degree of sensitivity, enabling the detection of subtle phenotypes that may be of significant medical value.

The phenotype derived from the knockout of a specific gene reveals both the potential therapeutic value as well as other target-specific side effects that may be anticipated for a small molecule inhibitor of that target. For instance, a target may display therapeutic potential in inflammation, but might also be critical for renal function. Without a mammalian knockout model, these deleterious target-specific side effects might not be observed until after significant amounts of time and resources have been spent on developing small-molecule compounds and testing them in preclinical or clinical development. When a drug produces a deleterious effect that was not observed in the knockout animal, it suggests that further optimization of the compound's specificity is worthwhile. The ability to produce strong preclinical data to support efficacy and lack of deleterious side effects for a novel target and corresponding lead compound further legitimizes the value of a drug discovery program and provides confidence to move ahead aggressively in development.

"The top 100 selling drugs in 2001 are only directed to some 29 drug targets, many with multiple agents addressing the same target. Of these 29 targets, 23 have been knocked out and in every case the knockout mouse was highly informative as to the on-target effects and side effects of the associated drugs."

Predicting breakthrough therapeutics

Gene knockouts can be viewed as modeling the biological mechanism of drug action by presaging the activity of highly specific antagonists *in vivo*. This information is critical when making decisions regarding target prioritization for a drug discovery enterprise. Since knockout mice have been shown to model drug activity, they provide an unprecedented level of predictive power over the drug discovery process and can be extremely valuable to the pharmaceutical and biotechnology industries. With the effective use of mouse knockout technology, expensive drug discovery activities such as high-throughput screening, medicinal chemistry, preclinical research and clinical trials can be focused on the drug targets that are most likely to lead to breakthrough therapeutics.

Hypothesis-driven gene targeting and gene trapping place physiology and therapeutic potential at the forefront of the drug discovery process and will provide primary data on the physiological function of virtually all members of 'drugable' gene families over the next few years. However, the full power of knockout mouse technology can only be realized when the predictive nature of knockout mouse phenotypes is applied early in the drug discovery process. The combination of mouse gene knockout technology and comprehensive physiological analysis will provide the pharmaceutical industry with novel, *in vivo*-validated targets with clear potential for the discovery of breakthrough therapeutics.

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FURTHER READING

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